CO-REGULATION OF RIBOSOMAL GENES IN

Neurospora crassa

by

Yuguang Shi

Plant Molecular Biology

Research School of Biological Sciences

The Australian National University

Canberra, AUSTRALIA

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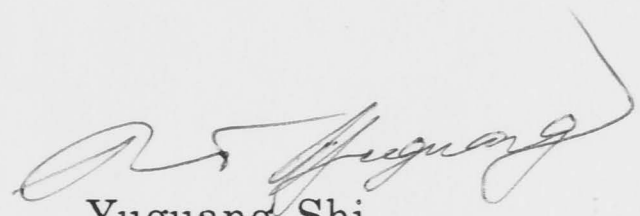
STATEMENT

The results presented in this thesis are my own original work and this thesis has not been submitted to any other institution or university for the award of any other degree. All source of information used in the preparation of this thesis are duly acknowledged in the text and in the References.



Yuguang Shi

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To

MOTHER

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ABSTRACT

Ribosomes, the major translation machinery, consist of 60-80 ribosomal proteins and 3-4 rRNAs. In eukaryotes, coordinated synthesis of the ribosomal components is believed to be implicated mainly at transcriptional level and is complicated by involvement of genes transcribed by three different RNA polymerase systems. The synthesis of rRNAs has been known to be coordinated with that of r-protein in a number of organisms including *Neurospora crassa*. However, little is known about the mechanisms involved. In *N. crassa*, a common *cis* element, the Ribo box, was found to be essential for transcription of both 5S and 40S rRNA genes, and three copies of this element occur in a r-protein gene (*crp-2*). Therefore, the Ribo box may play a role in coordination of transcription of ribosomal genes in *N. crassa*. This hypothesis was examined by experiments reported here.

The Ribo box was examined for its role in supporting formation of preinitiation complexes on 5S genes by the electrophoresis mobility shifting assay (EMSA) and by DNase I footprinting assay. It was demonstrated by EMSA that the Ribo box and the A box were not required for formation of a stable protein-DNA complex identified on a polyacrylamide gel. Instead, the C box played a predominant role in complex formation. In particular,

Ribo box alone was not capable in binding any specific transcription factors as detected by EMSA. The EMSA results were supported by competition experiments in which the 5S transcription was competed with DNAs from different 5S mutants. In the absence of the C box, neither the Ribo box, nor in combination with any other boxes, could compete for 5S transcription. However, DNase I footprinting experiments indicated that the Ribo box was required for formation of transcription complex on the 5S gene. The Ribo box, together with the A and C boxes, was protected from DNase I cleavage.

The Ribo box was functionally investigated in heterologous gene contexts, both *in vitro* in a gene transcribed by RNA polymerase III and *in vivo* in a gene transcribed by RNA polymerase II. The Ribo box was first examined for its interaction with the A box from a $tRNA^{Leu}$ gene in the 5S gene context. The 5S gene with the $tRNA^{Leu}$ A box was transcribed poorly *in vitro*, and the Ribo box and the C box were still required for transcription. The Ribo box was also tested in a tRNA-like context by constructing hybrids between a 5S gene and a $tRNA^{Leu}$ gene. It was demonstrated that in the tRNA-like context, the 5S A box supported a lower level of transcription than the tRNA A box, and the Ribo box was not required at all. Although the results suggest that all the 5S and tRNA internal control elements are gene specific in *N. crassa*, competition experiments demonstrated that at least one common transcription factor

was shared between the two genes. The Ribo box was also subjected to functional test *in vivo* by insertion into promoter of the *qa-2* gene which is transcribed by RNA polymerase II. The Ribo box insertion mutation was compared with a polylinker insertion for its effect on *qa-2* transcription. The results demonstrated that the *qa-2* mRNA level was not significantly altered by the Ribo box insertion in transformants grown under different nutritional conditions.

To look for sequences including the Ribo box involved in the transcriptional regulation of ribosomal protein genes in *N. crassa*, a ribosomal protein gene (*crp-3*) was cloned and sequenced. The *crp-3* gene is homologous to the yeast *rp51* and human *S17* genes. Sequence comparisons of the promoter regions of three sequenced *crp* genes including *crp-3* identified several conserved sequence elements which may potentially coordinate transcription of r-protein genes in *N. crassa*. In contrast to *crp-2* gene, no sequences with better than 4 mismatches to the Ribo box were found in the *crp-3* promoter. Using the *crp* genes as probes, plus a fourth ribosomal protein cDNA (*crp-4*), it was demonstrated by S1 nuclease mapping that the levels of the four ribosomal protein mRNAs were closely coordinated during a nutritional downshift from sucrose to quinic acid.

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

The ribosome is the major piece of machinery required for protein synthesis in living cells. It consists of two subunits, one small subunit (30S in prokaryotes, 40S in eukaryotes) which binds the mRNA and tRNAs, and one large subunit (50S in prokaryotes, and 60S in eukaryotes) which catalyzes peptide bond formation (Hill *et al.*, 1990). The ribosome is structurally and functionally conserved among prokaryotes and eukaryotes and requires multiple cellular components for assembly. These include 60-80 ribosomal proteins (r-proteins) and 3-4 rRNAs, a number which differs between prokaryotes and eukaryotes. Under the most favorable growth conditions, the ribosome constitutes as much as 40% of the total dried cellular mass, representing 85% and 15% of total cellular RNA and protein respectively (Mager, 1988; Nomura, 1986). With so much cellular energy required for ribosomal synthesis, coordinated synthesis of the multiple ribosomal components is essential.

Studies on ribosomal synthesis in *E. coli* have shown that free r-proteins and rRNAs are found in only very small quantities in growing cells. The rate of protein synthesis required for growth is achieved by controlling the number of ribosomes rather than modulating the activity of individual ribosomes (Nomura, 1986). In *E. coli* and yeast (*Saccharomyces cerevisiae*), ribosome synthesis is proportional to growth rate (Kief and Warner, 1981; Kjeldgaard and Gausing, 1974), and is promptly adjusted to changes in cellular growth and environmental conditions. (Nomura *et al.*, 1984; Warner, 1989).

Ribosomal synthesis can be regulated at different stages of the synthesis: transcriptional, post-transcriptional, translational, or assembly (reviewed by Nomura *et al.*, 1984; Warner, 1989). Studies on r-protein and rRNA genes in *E. coli* have demonstrated that the balanced synthesis of some 60 r-proteins and 3 rRNAs is primarily achieved by translational control (reviewed by Nomura *et al.*, 1984). In *E. coli*, the genes encoding most r-proteins and the rRNA precursors are organized as operons and certain r-proteins function as inhibitors of protein synthesis from their own mRNAs. This is known as autogenous control. In contrast, coordinated synthesis of ribosomal components in eukaryotes involves a substantial degree of transcriptional control, as in r-protein genes of yeast (reviewed by Planta and Raué, 1988). Translational control of r-protein synthesis has been observed in higher eukaryotes such as *Drosophila* (Kay

and Jacobs-Lorena, 1985; Schmidt *et al.*, 1985), *Xenopus* (Mariottini and Amaldi, 1990; Pierandrei-Amaldi *et al.*, 1985), and mouse (Kay and Jacobs-Lorena, 1985; Meyuhas *et al.*, 1987), but the regulation does not appear to be autogenous.

In eukaryotes, the genes encoding the ribosomal components are transcribed by three different RNA polymerases. The large rRNA precursors are synthesized by RNA polymerase I (pol I), the r-protein mRNA by RNA polymerase II (pol II), and the 5S rRNA by RNA polymerase III (pol III). Therefore the ribosome provides an intriguing system in which transcription by the three RNA polymerases must be co-ordinated to achieve a balanced supply of ribosomal components. The characteristics of each transcriptional system will be the major focus of this review, followed by general discussion on how the three RNA polymerase systems may interact with each other. Finally, possible mechanisms by which ribosomal genes in *N. crassa* may be co-regulated will be presented. Elucidation of this final point is the major objective of this thesis.

1.2. TRANSCRIPTION OF 40 rRNA PRECURSOR GENES BY THE RNA POLYMERASE I SYSTEM.

Pol I transcribes the more than 100 copies of the gene encoding the 40S

(45S in mammals) rRNA precursor only. This precursor is processed to the mature 18S, 28S, and 5.8S rRNAs of the ribosome (reviewed by Sollner-Webb and Tower, 1986). The 40 rRNA genes are clustered at one or a few chromosomal sites and organized as head-to-tail repeats among all eukaryotic organisms studied so far. Transcription by pol I can be distinguished from that by pol II and III by virtue of its resistance to high concentrations of the fungal toxin α-amanitin (Kedinger *et al.*, 1970). Studies on pol I transcription are more advanced in amphibians and mammalian systems than in lower eukaryotes such as yeast, largely because the first eukaryotic gene cloned was the 40S rRNA gene of *Xenopus* (Morrow *et al.*, 1974) and vertebrate *in vitro* transcription systems for pol I genes have been available for some time (Grummt, 1981).

Transcription of the human rRNA gene requires a bipartite promoter 5' to the coding region both *in vivo* and *in vitro* (Haltiner *et al.*, 1986; Jones *et al.*, 1988). One of the control elements is known as the UCE (upstream control element) located at -186 to -107 nt upstream from the initiation site. The other is the core element at -45 to +7 (Bell *et al.*, 1988). At least two transcription factors are required for active transcription in addition to the RNA pol I. These are the UBF (upstream binding factor) and SL1 (selectivity factor-1) (Bell *et al.*, 1988). Human UBF interacts with both the UCE and the core promoter elements. It is functionally exchangeable with the UBF from mouse but not with that from *Xenopus* (Bell *et al.*, 1990; Bell

et al., 1989; Learned *et al.*, 1985). The binding of human UBF protects a region between -75 and -114 in the UCE and weakly protects the core promoter region at -21 from DNase I cleavage in a footprinting assay (Bell *et al.*, 1988). The binding is significantly enhanced in the presence of SL1, resulting in extended protection of regions both upstream of the UCE from -115 to -165 and downstream of the core region from -21 to +1 site of initiation. Human SL1 does not interact specifically with the DNA template, and in the absence of UBF, SL1 together with RNA pol I can initiate a low basal level of transcription from both the full length promoter and the core promoter. Addition of UBF greatly enhances the transcription from the full length promoter, but only four-fold from the truncated promoter. Pol I transcription is strongly species specific and the specificity is conferred by SL1 rather than by UBF; addition of human SL1 to a mouse extract enables the extract to transcribe a human template efficiently (Learned *et al.*, 1985). SL1 also directs promoter specificity in reconstituted transcription reactions where purified transcription factors from human and mouse are mixed in different combinations (Bell *et al.*, 1990). Only when both SL1 and the promoter sequences originate from the same species does active transcription occur (Bell *et al.*, 1990). The transcription initiation site appears to be determined by UBF (Bell *et al.*, 1988). Human UBF has been purified to homogeneity and the gene encoding it has been cloned and characterized. The cloned gene contains DNA-binding motifs homologous to those found in high mobility group

protein 1 (HMG1) (Jantzen *et al.*, 1990).

In contrast to the human large rRNA gene, the *Xenopus* 40S promoter does not possess discrete domains in the 5' promoter region. Linker scanning mutations across the entire promoter reduce transcription (Reeder *et al.*, 1987; Windle and Sollner-Webb, 1986). In addition, a 60 or 81 bp repeated enhancer sequence in the spacer region between two rRNA repeats has been shown to be important for activation of transcription *in vivo* (Busby and Reeder, 1983; Moss, 1983; Windle and Sollner-Webb, 1986). The enhancer sequence is homologous to the -147 to +4 region of the 5' promoter sequence. *Xenopus* UBF binds to both the promoter and the enhancer sequences (Dunaway, 1989; Pikaard *et al.*, 1989). Similarly, a 140 bp repetitive sequence element in the mouse rRNA gene spacer region is required for transcriptional activation of the 40S gene both *in vivo* and *in vitro* (Kuhn *et al* ., 1990). In contrast to human SL1, mouse SL1 selectively binds tightly to both the UCE and the core promoter region, while UBF alone only binds weakly to the region between -88 to -108 even with saturating concentrations of factor (Bell *et al.*, 1990). In *S. cerevisiae*, because a faithful *in vitro* transcription system was lacking until recently (Lue and Kornberg, 1990; Riggs and Nomura, 1990), most of the studies on pol I transcription were carried out *in vivo* using rRNA minigenes (Planta and Raué, 1988). Promoter deletions assayed *in vivo* identified two regions important for 35S (35S in yeast) transcription. One is

a region from -149 to +15 that contains a bipartite promoter (-149 to -133 and -9 to +14) analogous to the mammalian 40S promoter. The other is an enhancer element located 2 kb upstream of the transcription initiation site. The yeast rRNA repeat unit differs from that of other organisms in that the 5S gene is located in the non-transcribed spacer region between 35S genes. This organization may facilitate the coordinated expression between the 40S and 5S genes, but there is still no direct evidence for this theory.

The rate of 45S rRNA transcription in mammalian cells is regulated by a variety of growth rate-related conditions, including nutritional starvation, glucocorticoids, cycloheximide, or serum withdrawal (Gokal *et al.*, 1986; Gokal *et al.*, 1990; Sollner-Webb and Tower, 1986). When cells are starved for essential amino acids or treated with cycloheximide, the levels of total mRNA and of rRNA are rapidly reduced. In the case of rRNA, this may be caused by reduced transcription rather than degradation, because an extract prepared from stationary phase cells is transcriptionally inactive (Buttgereit *et al.*, 1985; Gokal *et al.*, 1986; Sollner-Webb and Tower, 1986). The activity can be restored by addition of a protein fraction partially purified from extracts from log phase cells (Cavanaugh *et al.*, 1984; Cavanaugh and Thompson, 1985). The protein factor is a transcription factor known as TIF-IA whose level is regulated by growth rate (Schnapp *et al.*, 1990). TIF-IA co-purifies with pol I and is essential for transcription *in vitro*. TIF-IA may interact with pol I and convert it into a

transcriptionally active holoenzyme. The large subunit of the RNA pol I is heavily phosphorylated and dephosphorylation causes the polymerase to lose its specific activity in template recognition (Schnapp *et al*., 1990). It is possible that the polymerase is phosphorylated through direct interaction with the TIF-IA, but this has not been tested. Both r-protein and rRNA syntheses are reduced dramatically during the arrest of cell growth caused by starvation or hormonal treatment, which coincides with a rapid reduction of the TIF-IA level (Mager, 1988; Sollner-Webb and Tower, 1986). It is not known whether inactivation of TIF-IA is linked to repression of ribosomal gene transcription.

1.3. TRANSCRIPTION OF r-PROTEIN GENES BY THE RNA POLYMERASE II

In direct contrast to transcription initiated by the pol I and pol III, where only or a few different genes are transcribed by the two polymerases, RNA polymerase II is responsible for transcription of more than 10,000 genes encoding proteins. Among them are about 80 genes encoding ribosomal proteins, which under favorable growth conditions, account for 15% of the total cellular protein.

1.3.1. Promoter Elements That Bind Transcription Factors

Transcription by pol II is complicated by the diversity of genes to be

transcribed. Each gene is uniquely arranged as to number, type, and spatial array of *cis* elements required for transcriptional regulation. Yet, two basic DNA promoter elements are commonly used for transcription of many eukaryotic genes. These include the TATA box, and a sequence located at the transcription initiation site known as the initiator. The TATA box is an AT-rich region located 25-30 bp upstream of the initiation site of many mammalian genes (reviewed by Mitchell and Tjian, 1989). Its location in fungal genes is quite flexible, typically around -40 to -120 (reviewed by Struhl, 1989). The TATA box serves as the binding site for the general transcription factor TFIID, and is essential for efficient transcription of genes which carry the TATA box. However, there are a variety of genes that do not contain an apparent TATA box. For those genes, sequences which deviate substantially from the TATA consensus can serve as the binding sites for the TFIID (Ponticelli and Struhl, 1990; Singer *et al*., 1990). The initiator sequence is the primary determinant as to where transcription begins (Chen and Struhl, 1985; Johnson and McKnight, 1989).

In addition to the two basic promoter elements, the transcriptional effeciency of individual genes is strongly modulated by gene specific promoter elements. These elements are binding sites for positive or negative regulatory proteins. Some of these elements are common to many genes, such as the GC and CCAAT boxes among mammalian genes

(Mitchell and Tjian, 1989), and many upstream activation sequences (UAS) among yeast genes (Struhl, 1989)

1.3.2. General Transcription Factors

Transcription by pol II involves both general transcription factors which support basal levels of transcription, and gene specific transcription factors that are responsive to regulatory signals. At least six general transcription factors (TFs) are required in addition to pol II for transcription *in vitro*. These are TFIID, IIA, IIB, IIE, IIF, and IIG (reviewed by Sawadogo and Sentenac, 1990; Sumimoto *et al*., 1990). TFIID appears to be the only factor with an associated DNA binding activity among the six general TFs. Formation of the initiation complex begins with the binding of TFIID. The binding is stabilized by TFIIA, and is followed by the binding of TFIIB and RNA pol II. TFIIE and TFIIF appear to be incorporated into the complex via protein-protein contacts with pol II, a hypothesis which is supported by the fact that TFIIE and TFIIF can independently form a stable complex with RNA pol II (Flores *et al*., 1988; Flores *et al*., 1989). Human TFIIE has been purified to homogeneity and does not have any ATPase activity as previously suggested (Ohkuma *et al*., 1990). TFIIG, which is functionally related to TFIIA, stimulates transcription *in vitro* and is required for the assembly of a functional pre-initiation complex (Sumimoto *et al*., 1990), but its role in assembly is not yet clear.

1.3.3. Gene-Specific Transcription Factors

In addition to the general transcription factors, a large variety of gene-specific transcription factors and regulatory proteins have been identified (reviewed by Harrison and Aggarwal, 1990; Johnson and McKnight, 1989; Mermelstein *et al.*, 1989; Mitchell and Tjian, 1989; Struhl, 1989). Most of these are sequence-specific DNA binding proteins and share some common features. One of the common features is the bipartite protein structure consisting of two domains, one involved in protein-DNA interactions and the other involved in modulating transcription. The DNA binding domains of these transcription factors often share common protein structures or motifs such as "helix-turn-helix", "zinc finger", "leucine zipper", and "helix-loop-helix" (Harrison and Aggarwal, 1990; Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Ptashne, 1988; Struhl, 1989). However a few DNA binding proteins fit none of these categories (e.g. TFIID). The transcription effector domains also can be divided into several groups based on various common conserved features of the amino acid sequence, such as the acidic domain, glutamine-rich domain, and proline-rich domain (Mitchell and Tjian, 1989). Again there are certain transcription factors, such as RAP1/TUF, for which the transcription effector domain is poorly characterized. Several DNA binding and transcriptional effector motifs are conserved throughout evolution and are functionally exchangeable between higher and lower eukaryotes (reviewed

by Ptashne, 1988; Struhl, 1989).

Little is known about how specific transcription factors modulate transcription initiation. One way distal binding factors such as enhancer binding proteins may act is via DNA looping (reviewed by Ptashne, 1986). It is also not clear which of the general transcription factors and which steps in transcription initiation are the targets of transcriptional regulators. Comparisons between cloned and natural TFIID preparations in supporting transcriptional activation suggest the existence of previously unidentified proteins, variously called mediators (Kelleher *et al.*, 1990), co-activators (Kambadur *et al.*, 1990; Pugh and Tjian, 1990), or adaptors (Berger *et al.*, 1990) which mediate the interaction between activator proteins and TFIID. It is suggested that the co-activator is the target site for activators (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Pugh and Tjian, 1990), while TFIIB has been shown recently to be a direct target of an acidic activator protein (Lin and Green, 1991). It is likely that all the general transcription factors are potential target sites for activator (or repressor) proteins either directly or via mediators. In each case, the RNA pol II must be the ultimate target for activation or repression by the gene specific factors.

1.3.4. Co-Ordinate Regulation of Gene Families

In contrast to the prokaryotic paradigm that functionally related

genes are usually jointly regulated by their inclusion in operons, functionally related genes in eukaryotes are often scattered around the genome. Eukaryotic genes whose expression responds to common intra- and extra-cellular cues are often co-regulated via a transcription factor(s) whose recognition sites are common among the genes. Examples are genes responsive to heat shock, hormones, and growth factors in mammalian cells, and genes responsive to nutritional states in lower eukaryotes such as yeast and *N. crassa*. In the yeast *S. cerevisiae*, expression of genes encoding amino acid biosynthetic enzymes by different pathways are coordinately regulated via a common activator protein known as GCN4 (Hope and Struhl, 1985). In response to amino acid starvation, the GCN4 protein selectively binds to the promoter regions of many amino acid biosynthetic genes and activates their transcription (Arndt and Fink, 1986).

1.3.5. Co-Regulation of Ribosomal Protein Genes

Transcription of yeast ribosomal protein (r-protein) genes is coordinated under a variety of conditions including carbon or nitrogen nutritional shifts (Kief and Warner, 1981; Kraig *et al.*, 1982; Pearson and Haber, 1980; Warner and Gorenstein, 1978; Warner and Gorenstein, 1977). Analysis of the 5' flanking sequences of more than 20 r-protein genes revealed a 15 bp conserved sequence, the UAS_{rpg} (reviewed by Mager, 1988; Planta and Raué, 1988; Warner, 1989). The UAS_{rpg} is located in single or

duplicated copies 250-450 bp upstream of the AUG in all but two (*L3* and *S33*) of the yeast r-protein genes studied so far. Promoter deletion and gene fusion studies of several genes possessing the sequence confirmed that the UAS_{rpg} is responsible for the coordinate activation of r-protein genes during a nutritional upshift (Donovan and Pearson, 1986; Herruer *et al.*, 1987; Rotenberg and Woolford, 1986; Teem *et al.*, 1983). The UAS_{rpg} also can activate transcription when placed downstream of a coding region, but with low efficiency (Guarente and Hoar, 1984). The UAS_{rpg} also is found 5' to the gene encoding a common subunit of RNA pol I and pol III (Mann *et al.*, 1987), suggesting that the UAS_{rpg} might coordinate the synthesis of r-proteins with that of the rRNAs in yeast. However, this has not yet been tested, and the UAS_{rpg} is also found in many other genes not associated with protein synthesis (Warner, 1989). A regulatory protein variously known as TUF, RAP or GRF which binds to the UAS_{rpg} has been identified and characterized (Buchman *et al.*, 1988a; Buchman *et al.*, 1988b; Huet and Sentenac, 1987; Shore and Nasmyth, 1987; Shore *et al.*, 1987). The TUF protein is acidic and contains high proportion of asparagine residues. It does not contain any common structural motifs classified from other DNA binding proteins, such as zinc finger or leucine zipper (Johnson and McKnight, 1989; Shore and Nasmyth, 1987)

1.4. TRANSCRIPTION OF 5S rRNA GENES BY THE RNA POLYMERASE III SYSTEM

The major difference between transcription by pol III and that of other two polymerase systems is the employment of strong internal promoters in the case of RNA pol III. In comparison to pol II, relatively few genes, all of which encode small RNAs, are transcribed by pol III. These include genes coding for 5S rRNA (for ribosomal synthesis), tRNAs (for protein translation), U6 snRNA (for mRNA processing), 7SL RNA (for protein transport), VA RNA (encoded by the adenovirus genome for viral regulation), and for few RNAs with unknown function such 7SK, M, human Alu I, rodent B2, and salmon SINE RNAs (see review by Geiduschek and Tocchini-Valentini, 1988; Palmer and William, 1990). The transcription of both 5S and tRNA genes has been studied in detail in a number of organisms. Early studies on *Xenopus* 5S and tRNA genes demonstrated that the internal control region (ICR) alone was sufficient for efficient transcription both *in vitro* and *in vivo*, though recent evidence shows that the 5' flanking sequence also modulates transcriptional efficiencies (Oei and Pieler, 1990). Like the large rRNA genes, the 5S genes are repeated many times in eukaryotic genomes either in tandem such as in *Xenopus*, or in scattered positions such as the 5S genes of *N. crassa*.

5S genes from different organisms differ in the structure and

organization of the internal elements, although some of the elements are conserved, namely the A and C boxes. In *Xenopus*, the 5S ICR is composed of three distinct sequence elements, the A box, the intermediate box and the C box localized at +50 to +64, +67 to +72, and +80 to +97 respectively (Pieler *et al.*, 1987). Even though the yeast 5S gene carries sequences homologous both to A and C boxes of *Xenopus*, it has been shown by Challice and Segall (1989) that only the C box plus a promoter element at +1 is essential for transcription *in vitro*. In *Drosophila* (Sharp and Garcia, 1988), silkworm (Morton and Sprague, 1984), and *N. crassa* (Tyler, 1987), additional internal elements are required for efficient transcription *in vitro*. By comparison, all eukaryotic tRNA genes share similar structures (reviewed by Sharp *et al.*, 1985). These include an A and B box, and the spacing between the two boxes is very flexible (reviewed by Geiduschek and Tocchini-Valentini, 1988). In addition to the internal sequences, the -10 region and upstream flanking sequences of the *Xenopus* 5S gene are also important in modulating transcription efficiency *in vitro* (Oei and Pieler, 1990). In yeast, 50 bp of the 5' flanking sequences were protected from DNase I cleavage during transcription initiation *in vitro,* as well as the ICR (Braun *et al.*, 1989). In other organisms, such as *Drosophila* (Sharp and Garcia, 1988), silkworm (*B. mori*) (Morton and Sprague, 1984), and *Neurospora crassa* (Tyler, 1987), 5' flanking sequences including TATA-like sequences are also important. Some pol III transcribed genes, such as the human U6 snRNA, require only 5' flanking

sequences (Das *et al.*, 1988; Murphy *et al.*, 1987), while yeast U6 gene also requires sequences 3' to the coding region (Brow and Guthrie, 1990).

Transcription of 5S rRNA and tRNA genes shares some common features. Both genes have internal promoter sequences and share transcription initiation factors TFIIIC and TFIIIB (see Geiduschek and Tocchini-Valentini, 1988; Palmer and William, 1990 for review). Yet, the way in which these common transcription factors are assembled on the promoters must be different. Transcription of 5S genes requires a 5S specific internal element, the C box, which is the major binding site for transcription factor TFIIIA. The binding of TFIIIA is a prerequisite for subsequent binding of TFIIIC, TFIIIB, and pol III. The role of the other internal element of 5S genes, the A box, is less well understood. In *Xenopus*, TFIIIA alone makes some contacts with the A box, and also, certain A box mutations interfere with the binding of TFIIIC to the TFIIIA.5S DNA complex (Pieler *et al.*, 1987). But no binding of TFIIIC to the A box can be detected by DNaseI footprinting (Carey *et al.*, 1986; Fradkin *et al.*, 1989). In yeast, the A box is protected from DNase I only in the presence of all three factors; TFIIIA, TFIIIC and TFIIIB (Kassavetis *et al.*, 1990). In contrast, transcription of tRNA genes involves direct binding of TFIIIC to an element called the B box which is found in all tRNA genes but not in 5S genes (Geiduschek and Tocchini-Valentini, 1988; Sharp *et al.*, 1985). tRNA genes also contain an A box which shares

sequence homology to the 5S A box. In a *Xenopus* oocyte transcription assay, the A boxes of a 5S and a tRNA gene appeared to be functionally exchangeable (Ciliberto *et al.*, 1983). However, TFIIIC alone clearly contacts the tRNA A box following binding to the B box (Baker *et al.*, 1986; Carey *et al.*, 1986; Kassavetis *et al.*, 1990). Binding of TFIIIC is followed by the binding of TFIIIB and RNA pol III. It is not known how TFIIIC participates in the different types of initiation complexes found on the 5S and tRNA genes.

Using partially purified pol III transcription factors, it was demonstrated that in yeast, TFIIIA and TFIIIC are assembly factors while TFIIIB alone can initiate multiple rounds of transcription (Kassavetis *et al.*, 1990). However, the lack of any cloned yeast pol III transcription factors has hindered the examination of more complex problems, such as how the transcription factors interact with each other. TFIIIA is the first eukaryotic factor cloned due to its abundance in *Xenopus* oocytes (reviewed by Geiduschek and Tocchini-Valentini, 1988). TFIIIA carries 9 repeats of a 30-amino acid sequence that is responsible for DNA binding (Brown *et al.*, 1985; Miller *et al.*, 1985b). These repeats contain a DNA binding motif,"zinc fingers", which consist of pairs of cysteine and histidine residues. Using a truncated TFIIIA synthesized *in vitro*, it was demonstrated that the cluster of zinc fingers contacts three parts of the ICR that coincide with the promoter elements, with highest

affinity to the C box element (Vrana *et al.*, 1988). TFIIIC has been extensively purified in a number of systems (Fradkin *et al.*, 1989; Johnson and Wilson, 1989). Yeast TFIIIB has been purified to apparent homogeneity (Klekamp and Weil, 1986). Cloning of genes for these factors and reconstitution of the factors from subunits expressed in *E. coli* will facilitate further studies on many aspects of transcription by pol III system.

1.5. RELATIONSHIPS AMONG THE THREE RNA POLYMERASE SYSTEMS

Although the three RNA polymerases employ distinct transcription systems, the three systems share some common features. One of these common features is the polymerases themselves. The three RNA polymerases each consist of multiple subunits and these subunits share homology. One of these is the largest subunit which contains DNA binding domains and is phosphorylated in all three polymerases (reviewed by Geiduschek and Tocchini-Valentini, 1988; Palmer and William, 1990; Saltzman and Weinmann, 1989; Sawadogo and Sentenac, 1990; Schnapp *et al.*, 1990). Phosphorylation and dephosphorylation are important steps involved in signal transduction and cell cycle determination, as well as global control of living cells in response to growth conditions (Klausner and Samelson, 1991; Lewin, 1990; Nurse, 1990). It has been shown that

dephosphorylation of pol I abolishes its specific activity in transcription *in vitro* without significantly affecting its transcription of nonspecific templates (Schnapp *et al.*, 1990). It is possible that phosphorylation is one of the mechanisms involved in coordinating the activities of the three RNA polymerases.

There are some similarities shared between the pol I and pol II transcription systems. These include the fact that both polymerases can use a minimal promoter in the immediate vicinity of the transcription start site (core promoter). Both system utilize enhancers which stimulate transcription at a distance. In the yeast system, some enhancer elements from RNA pol I and pol II transcribed genes are functionally interchangeable (Lorch *et al.*, 1990). For example, the yeast 35S enhancer binding protein REB1 also recognizes promoter sequences located in several genes transcribed by RNA pol II (Ju *et al.*, 1990).

Transcription by pol III also shares some common features with pol II. Some cellular and viral protein coding genes require internal sequences for efficient transcription (Murphy *et al.*, 1989). 5' sequences are important for modulating transcription of some 5S and tRNA genes as in pol II promoters (Murphy *et al.*, 1989; Oei and Pieler, 1990). Some pol III genes, such as U6 and 7SK genes, employ only the 5' promoter sequences (Carbon *et al.*, 1987; Murphy *et al.*, 1987). The mammalian U6 and 7SK

genes share at least two promoter elements with U1 and U2 snRNA genes which are transcribed by pol II (Carbon *et al.*, 1987; Murphy *et al.*, 1989), though they may bind different transcription factors. Recent evidence has demonstrated that the general pol II transcription factor, TFIID, is required for transcription of yeast U6 gene which is transcribed by pol III (Margottin *et al.*, 1991).

1.6. CO-REGULATION OF rRNA and r-PROTEIN GENES IN *N. crassa*: MODELS AND OBJECTIVES

Transcription of 5S genes in *N. crassa* requires A and C boxes similar to the *Xenopus* ICR, plus a TATA sequence at -29 which directs the startpoint of transcription (Tyler, 1987). It also requires an extra internal element at +18 to +30, the Ribo box, which shares no homology with any internal control elements of 5S genes in other systems (Tyler, 1987). The Ribo box is essential for transcription *in vitro* and a two bp substitution within the Ribo box reduces transcription 4-fold (Tyler, 1987). In contrast to the 5S genes in *Xenopus* and yeast, the spacing between the internal elements is very strict, a 4 bp insertion or deletion between any two internal elements (the Ribo, A, and C boxes) dramatically reduces the transcriptional efficiencies *in vitro* (Tyler, 1987).

Transcription of the *N. crassa* 40S rRNA gene *in vitro* by pol I

requires a bipartite promoter consisting of domains I (-113 to -36) and II (-28 to +4) (Tyler, 1990). This arrangement is similar to the 40S gene in *Xenopus* in which all sequences between -140 and +10 except -82 to -77 are essential for full transcription (Reeder *et al.*, 1987). Domain II is also similar to the core promoter in human and mouse and contains a TATA box at -5. A two base pair substitution within the TATA box reduces transcription 50-fold (Tyler, 1990). In particular, domain I contains sequences homologous to the 5S Ribo box and mutations within the 40S Ribo box region cause a severe reduction in transcription of the 40S gene *in vitro* (Tyler, 1990). The 40S Ribo box has been shown to be functionally exchangeable with that of the 5S gene (Tyler and Harrison, 1990). This suggests that the Ribo box may coordinate transcription of the 40S gene with that of the 5S gene.

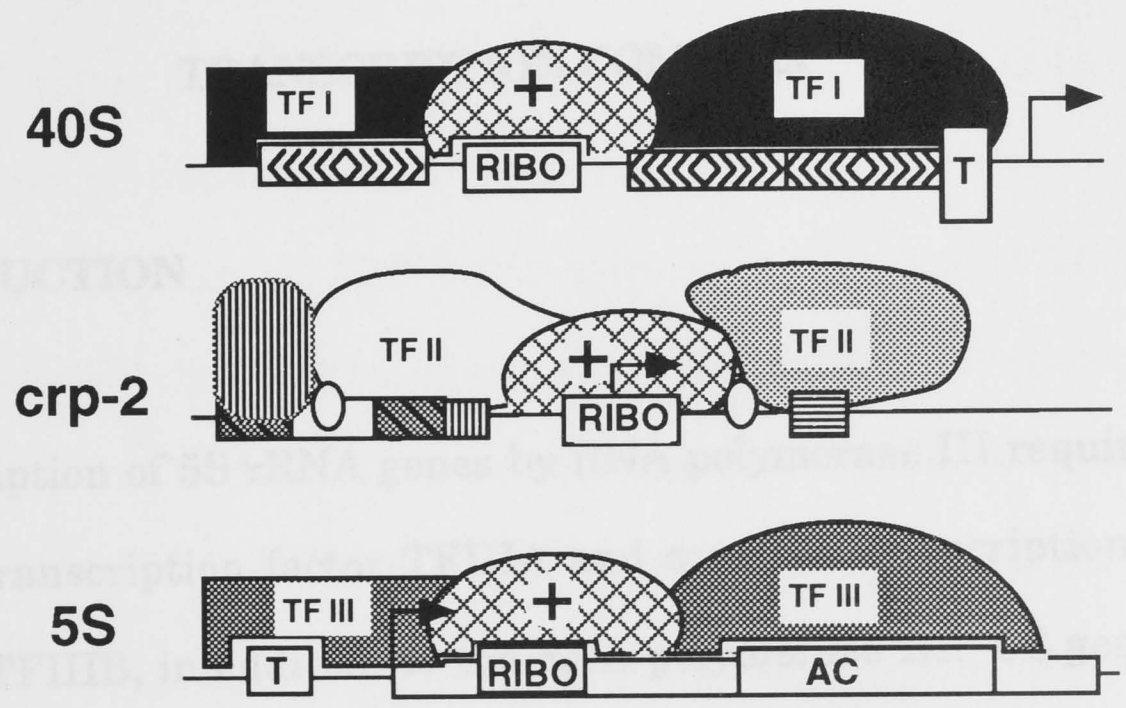
In *N. crassa*, the synthesis of rRNA is coordinated with that of r-proteins *in vivo*. During a nutritional upshift from acetate to glucose, the rates of rRNA and r-protein synthesis increase markedly within 30 min of the shift. During a downshift from glucose to glycerol, both rRNA and r-protein synthesis are severely inhibited for at least two hours after the shift before a slower rate of synthesis recommences (Alberghina *et al.*, 1975; Alberghina *et al.*, 1978; Sturani *et al.*, 1976; Sturani *et al.*, 1973). However, little is known about the mechanisms involved at either the transcriptional or translational levels.

Based on *in vitro* transcription studies of *N. crassa* 5S and 40S genes, it has been hypothesized that the Ribo box may play a role in coordinating transcription of the rRNA genes. Three copies of the Ribo box sequence also are present in the 5' flanking region of a ribosomal protein gene *crp-2* (Tyler and Harrison, 1990). One of the Ribo boxes is located at the transcription initiation site of the *crp-2* gene. Therefore the Ribo box might also coordinate transcription of the rRNA with the r-protein genes by serving as a binding site for a common transcription factor. This factor could be an activator, like the GCN4 and TUF proteins in yeast (Arndt and Fink, 1986; Warner, 1989). Under this hypothesis, a nutritional upshift could cause a change in the activity of the activator, which in turn would promote the binding of the factor to the Ribo box, resulting in elevated transcription of the three genes (Fig 1A). Alternatively, the Ribo box binding factor could be a co-repressor and the binding of the factor would repress transcription of these genes in response to a nutritional downshift. Both hypotheses are tested through the experiments presented in this thesis.

FIG. 1. Models of the possible role of the Ribo box in coordinating transcription of ribosomal genes in *N. crassa*. A: a positive regulatory model in which the binding of a common activator to the Ribo box activates transcription of all three ribosomal genes. B: a negative regulatory model in which the binding of a common repressor to the Ribo box abolishes transcription of the three genes. Arrows indicate the transcription initiation site. TF: Transcription factor; RIBO: Ribo box; T: TATA box; AC: A and C box of the 5S gene.

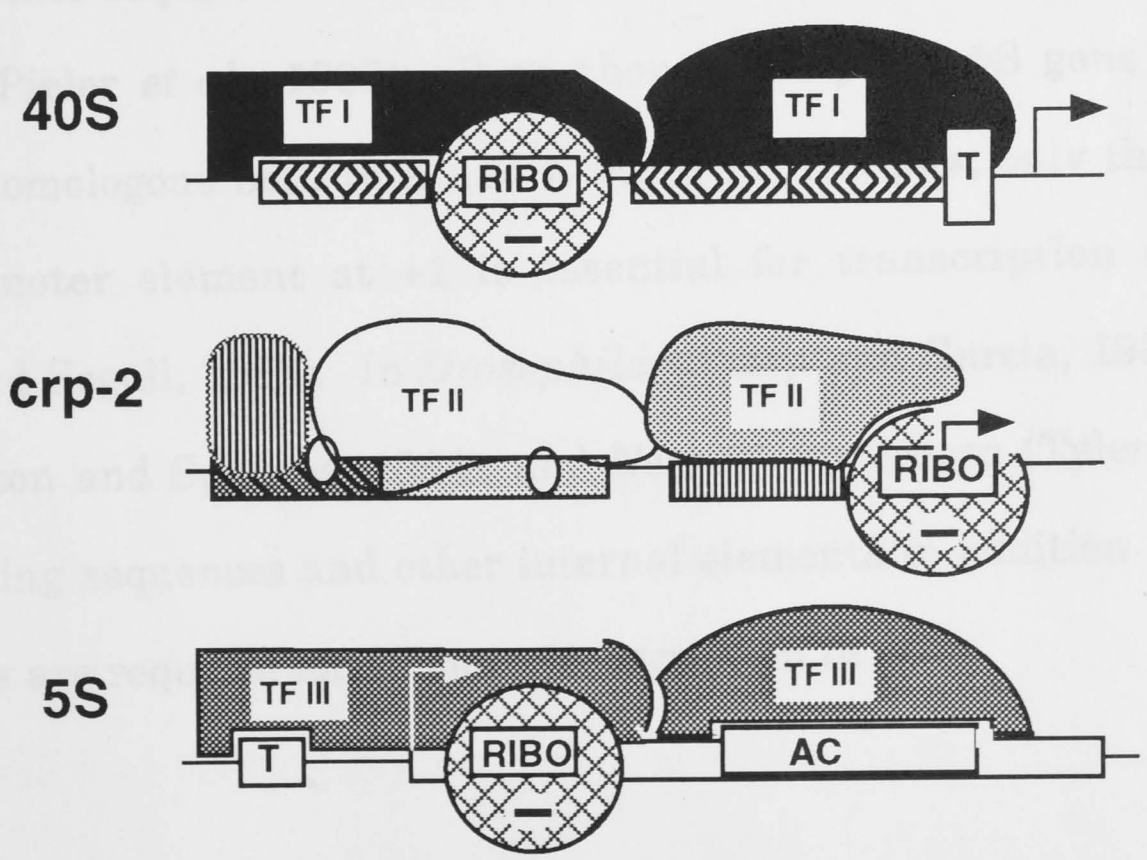
A

POSITIVE REGULATORY MODEL



B

NEGATIVE REGULATORY MODEL



CHAPTER 2

EFFECTS OF PROMOTER MUTATION ON FORMATION OF THE 5S TRANSCRIPTION COMPLEX

2.1. INTRODUCTION

Transcription of 5S rRNA genes by RNA polymerase III requires both 5S specific transcription factor TFIIIA and common transcription factors TFIIIC and TFIIIB, in addition to the RNA polymerase III. 5S genes from different organisms differ in structure and organization of the internal elements, though some of the elements are conserved such as the A and C boxes. In *Xenopus*, the 5S gene internal control region (ICR) is composed of three distinct sequence elements, the A box, the intermediate box and the C box (Pieler *et al.*, 1987). Even though the yeast 5S gene carries sequences homologous both to A and C boxes in *Xenopus*, only the C box plus a promoter element at +1 is essential for transcription *in vitro* (Challice and Segall, 1989). In *Drosophila* (Sharp and Garcia, 1988), silk worm (Morton and Sprague, 1984), and *Neurospora crassa* (Tyler, 1987), the 5' flanking sequences and other internal elements in addition to the A and C boxes are required for efficient transcription *in vitro*.

Formation of transcription initiation complexes on 5S genes involves the sequential binding of transcription factors TFIIIA, C, and B to the DNA template. TFIIIA has been shown by both DNase I footprinting and methylation interference assays to bind most of the internal control region (ICR) of the *Xenopus* 5S gene with the highest affinity at the C box region. Some single base pair substitutions in the region greatly decrease or totally abolish binding (Pieler *et al.*, 1987). The subsequent binding of TFIIIC and TFIIIB leaves little or no additional signature within the ICR (Carey *et al.*, 1986; Fradkin *et al.*, 1989). In yeast 5S genes the pattern of DNase I protection assay is very different. TFIIIA alone protects only the C box region; it does not protect the A box region, even in conjunction with TFIIIC (Kassavetis *et al.*, 1990). Incorporation of TFIIIC protects a region downstream of the C box. Subsequent addition of TFIIIB greatly extends the interaction along the 5S gene, so that the entire ICR plus 43 bp upstream from the initiation site is protected from the DNase I cleavage (Braun *et al.*, 1989; Kassavetis *et al.*, 1990).

The *N. crassa* 5S promoter consists of three internal elements and a TATA box at -29 based on *in vitro* transcription studies (Tyler, 1987). The internal elements resemble the A and C boxes found in *Xenopus*, plus a third element, called the Ribo box, which has not been found in the 5S genes of other organisms. The TATA box of the *N. crassa* 5S gene has been shown to direct the start point of transcription (Tyler, 1987). In contrast to

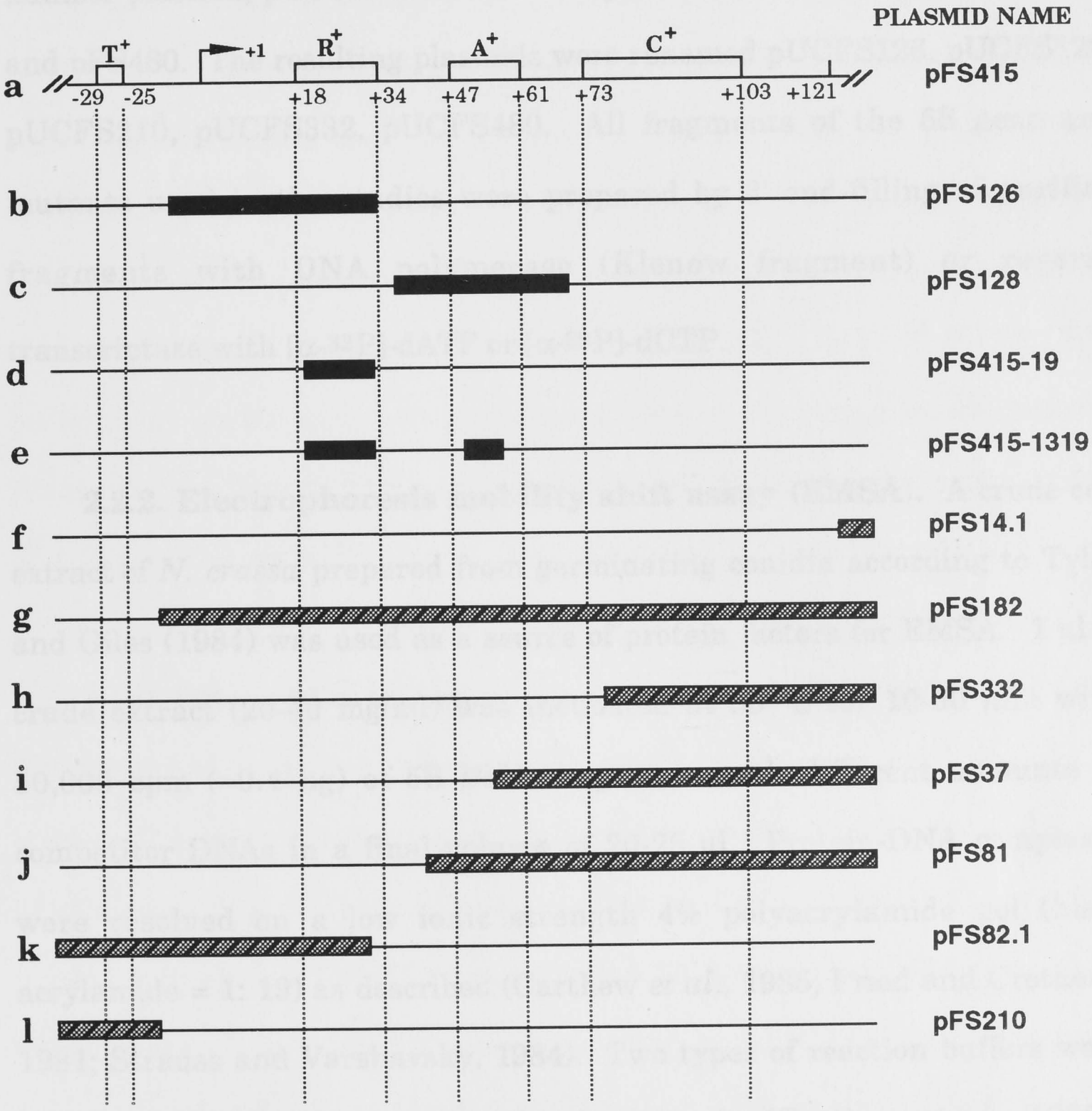
the 5S genes of *Xenopus* and yeast, the spacing between the internal elements is very strict, a 4 bp insertion or deletion between any two internal elements (the Ribo, A, and C boxes) dramatically reduces the transcription efficiency (Tyler, 1987) *in vitro*. The Ribo box is essential for transcription *in vitro* and a 2 bp substitution within the Ribo box reduces transcription 4 fold (Tyler, 1987). Additionally, the Ribo box is required for transcription of the 40S rRNA gene by RNA polymerase I (Tyler, 1990), and is found three times in the 5' flanking region of the r-protein gene *crp-2* transcribed by the RNA polymerase II (Tyler and Harrison, 1990). Therefore, the Ribo box might be involved in coordinating the transcription of the three different categories of ribosomal genes. Unlike the 5S gene in *Xenopus* and yeast, there is little information on how each internal element of *N. crassa* 5S gene may be involved in the formation of transcription initiation complex, particularly whether the Ribo box interacts with any transcription factors, general or Ribo box-specific.

This chapter describes studies on the role of internal promoter elements of the *N. crassa* 5S rRNA gene in supporting the formation of transcription complexes using the following methods: electrophoresis mobility shift assay (EMSA), DNase I footprinting assay, and transcription competition assay. Specific attention is paid to identifying trans-acting factors that interact with the Ribo box.

2.2. MATERIALS AND METHODS

2.2.1. Plasmids and probes. The plasmids used here carry either the wild type α 52 5S gene (Selker *et al.*, 1981) or mutants of it. Plasmid pFS426 carries the entire wild type 5S gene plus 230 bp of 5' flanking sequences (Fig 1a). Plasmid pFS14.1 carries a 5S gene without termination sequences (3' deletion to +120) (Fig 1f) and was used as a maxigene (Lasser *et al.*, 1983; Segall, 1986) in transcription competition experiments. Plasmids which carry the 5S deletion mutants are: pFS182, a 3' deletion mutant covering the entire ICR downstream of the TATA box (Fig 1g); pFS332, a 3' deletion mutant covering the C box downstream of +76 (Fig 1h); pFS37, a 3' deletion mutant covering the C box downstream of +57 (Fig 1i); pFS81, a 3' deletion mutant covering the A and C boxes downstream of +40 (Fig 1j); pFS82.1, a 5' deletion mutant covering the Ribo box upstream of +28 (Fig 1k); and pFS210, a 5' deletion mutant covering the TATA box upstream of -21 (Fig 1l). The plasmids which carry 5S substitution mutants are: pFS126, which carries a large region of base substitution covering both the Ribo box and the immediate upstream regions (Tyler, 1987) (Fig 1b); pFS415-19 which carries a 12 bp substitution within the Ribo box (Chapter 3; Shi and Tyler, 1991) (Fig 1d); pFS128 which carries a large region of base substitution spanning the entire A box and the surrounding sequences (Tyler, 1987) (Fig 1c); and pFS415-1319 which carries a 12 bp substitution within the Ribo box and a 5 bp substitution within the A box to match the A

FIG. 1. Structures of wild-type and mutant 5S genes used in Chapter 2. T, R, A and C indicate the TATA, Ribo, A and C boxes respectively. +1 indicates the start point of transcription. Black boxes indicate substitution mutations and shaded boxes indicate deletion mutations. Positions of the 5S elements are from Tyler (1987).


PLASMID NAME
T+
+1
R+
A+
C+
a
-29
-25
+18
+34
+47
+61
+73
+103
+121
pFS415
b
pFS126
c
pFS128
d
pFS415-19
e
pFS415-1319
f
pFS14.1
g
pFS182
h
pFS332
i
pFS37
j
pFS81
k
pFS82.1
l
pFS210

box consensus of tRNA genes (Chapter 3; Shi and Tyler, 1991) (Fig 1e). In order to isolate enough DNA for competition analysis in EMSA, some of the 5S fragments carried on pBR322 vectors were sub-cloned into the high copy number plasmid, pUC18. These include pFS126, pFS128, pFS210, pFS332, and pFS480. The resulting plasmids were renamed pUCFS126, pUCFS128, pUCFS210, pUCFS332, pUCFS480. All fragments of the 5S gene and mutants used in the studies were prepared by 3' end-filling of purified fragments with DNA polymerase (Klenow fragment) or reverse transcriptase with [α-^{32}P]-dATP or [α-^{32}P]-dCTP.

2.2.2. Electrophoresis mobility shift assay (EMSA). A crude cell extract of *N. crassa* prepared from germinating conidia according to Tyler and Giles (1984) was used as a source of protein factors for EMSA. 1 µl of crude extract (20-40 mg/ml) was incubated at 30 °C for 10-30 min with 50,000 cpm (~0.4 ng) of 5S DNA fragments with different amounts of competitor DNAs in a final volume of 20-25 µl. Protein-DNA complexes were resolved on a low ionic strength 4% polyacrylamide gel (bis : acrylamide = 1: 19) as described (Carthew *et al.*, 1985; Fried and Crothers, 1981; Strauss and Varshavsky, 1984). Two types of reaction buffers were used. Buffer 1, adapted from Carthew *et al.* (Carthew *et al.*, 1985), contained 12 mM Hepes-KOH (pH7.9), 60 mM KCl, 5mM $MgCl_2$, 4mM Tris-HCl (pH7.9), 0.6 mM EDTA, 0.6 mM DTT, and 12% glycerol. Buffer 2

was the same as the *in vitro* transcription buffer (Tyler and Giles, 1984) with the addition of 12% glycerol, and contains 10 mM KHepes (pH 7.9), 5 mM K_2EGTA, 103 mM K-acetate, 10 mM Mg acetate, and 2.5 mM DTT. Competitor DNA was either poly (dI-dC) (Pharmacia), plasmid DNA (pUC18), calf thymus DNA, or fragments containing 5S sequences. Gels were pre-electrophoresed for 80 min at 20mA, and the protein-DNA complexes were resolved by electrophoresis at room temperature until the bromophenol blue reached the bottom of the gel. The running buffer contained 6.7 mM Tris-HCl (pH7.9), 3.3 mM NaoAc, and 1mM EDTA. The buffer was circulated between compartments during electrophoresis. After electrophoresis, gels were transferred to Whatman 3M paper, dried and autoradiographed.

2.2.3. DNase I Footprinting. Footprinting was performed according to Carthew *et al*. (Carthew *et al*., 1985), with modifications. Briefly, 1 µl of crude cell extract was incubated with approximately 50,000 cpm (~0.4 ng) of wild type 5S (pFS415) or Ribo box mutant (pUCFS126) fragments in buffer 1, plus 3-10 µg poly (dI-dC) or calf thymus DNA as carrier DNA. The final volume was 20 µl. After 20 min incubation at 30 °C, the reactions were treated with either 2 µl of 0.5 µg/µl DNase I without $CaCl_2$ or 2 µl of 15 ng/µl DNase I containing 20 mM of $CaCl_2$ for 60 sec at room temperature. For DNase I digestion of DNA in the absence of extract, 2 µl of 50 ng/µl (or 6

ng/μl with 20 mM $CaCl_2$) of DNase I were used in the digestion. The DNase I digestions were stopped by adding EDTA to 50 mM, followed by extraction with an equal volume of each of phenol and chloroform, and precipitated with 3 volume of ethanol. The DNA was denatured at 95 °C for 2 min then applied to a standard 8% polyacrylamide 7 M urea sequencing gel.

2.2.4. Transcription competition assay. *In vitro* competition of 5S transcription with wild type and mutant 5S gene was conducted as described by Shi and Tyler (1991) with a slight modification. Transcription reactions for different competition reactions were set up together on ice before aliquoting into tubes containing different competitor DNAs, and then transferred to 30 °C for 20 min as usual.

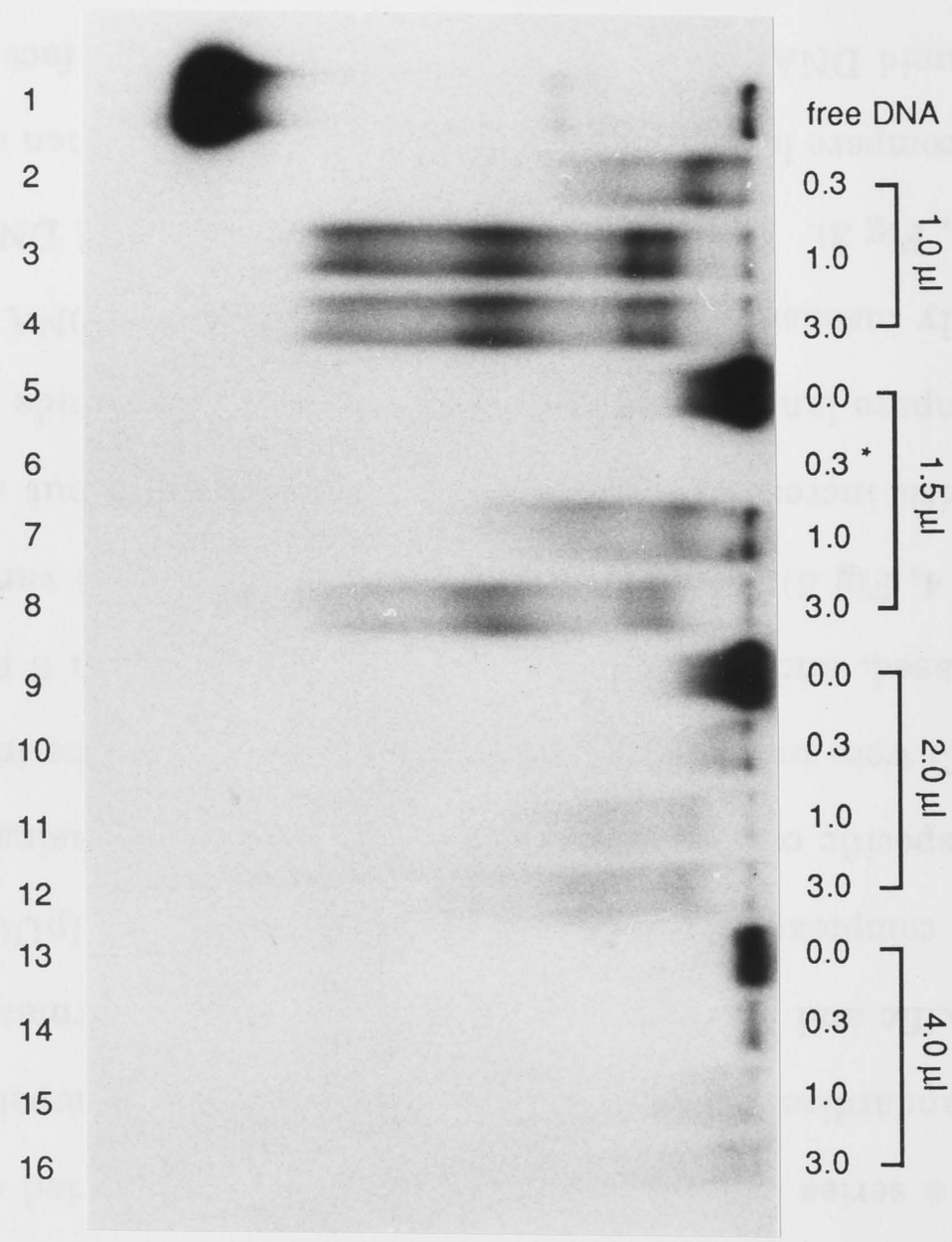
2.3. RESULTS

2.3.1. Formation of protein-DNA complexes on the 5S rRNA gene. The electrophoresis mobility shift assay (EMSA) was used to detect factors that specifically recognize the internal elements and the upstream region of the *N. crassa* 5S gene. EMSA is based on the observation that DNA fragments that are bound to proteins migrate through low-ionic-strength polyacrylamide gels more slowly than free DNA fragments. Certain amounts of carrier DNA must be used to titrate non-specific DNA binding

proteins which are present in abundance in crude extracts. However, care has to be taken to titrate the quantity of carrier DNA used, because specific DNA binding factors also have a low affinity for non-specific DNA. The carrier DNA also serves as an inhibitor of nucleases present in the extract.

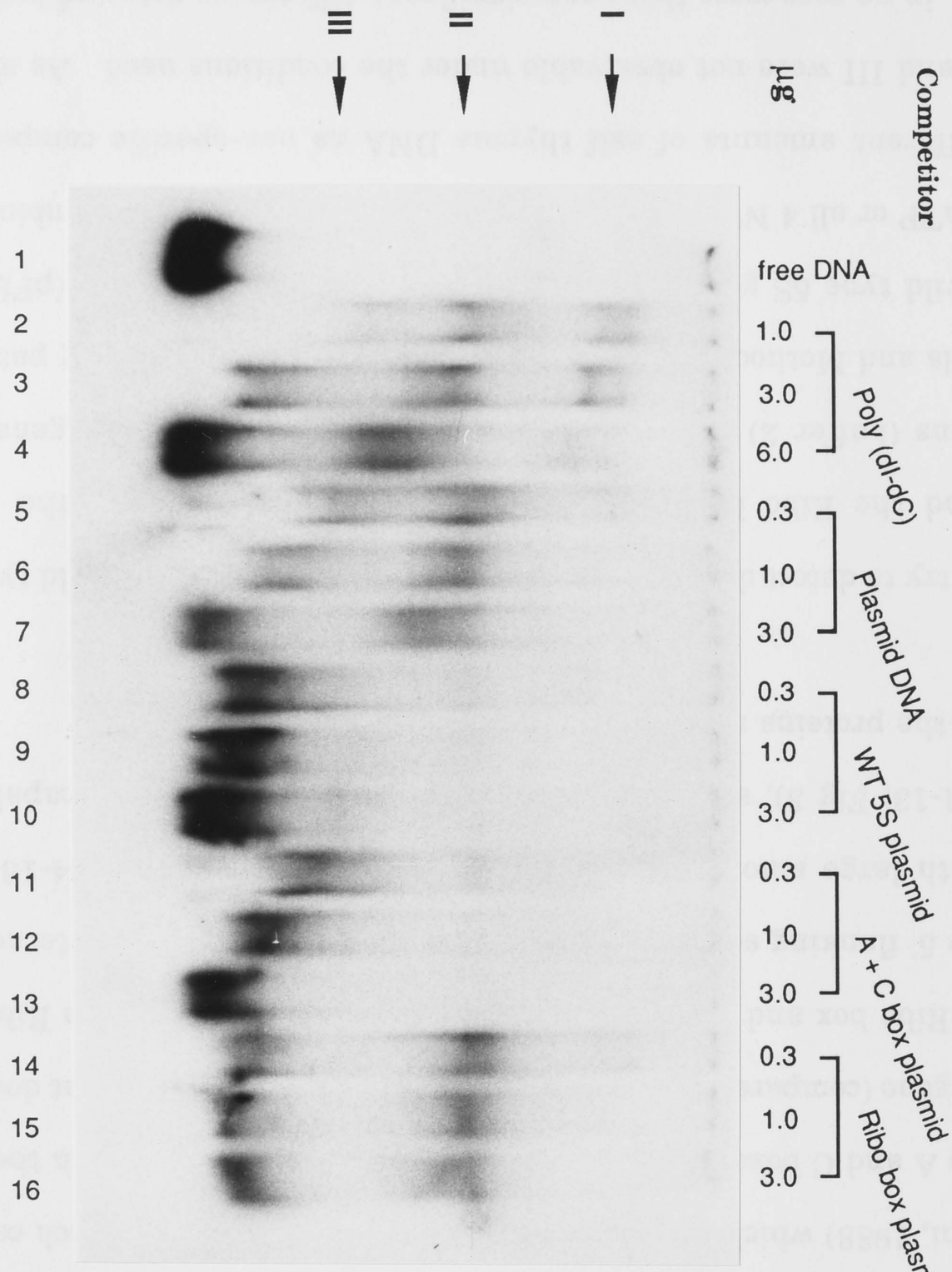
To determine the ratio of carrier DNA to crude cell extract, different amounts of carrier DNA were included with varying amounts of extract. Poly (dI-dC) was used as carrier DNA based on its weak ability to compete for specific DNA binding factors (Carthew *et al.*, 1985). The DNA probe used in the studies was a 230 bp wild type 5S fragment from pFS415 carrying the entire ICR plus the upstream region including the TATA box. Three separate retarded bands could be identified with certain ratios of carrier DNA and extract (Fig 2). Fig 2 shows that three different ratios of carrier DNA to extract produced well separated retarded bands (lane 3,4, and 8 in Fig 2). These ratios were 1 µl extract with 1 µg or 3 µg poly (dI-dC), and 1.5 µl of extract with 3 µg of poly (dI-dC). When no carrier DNA was used, the labelled DNA would not migrate from the well into the gel due to binding of the probe by non-specific DNA binding proteins (lanes 5, 9, 13 in Fig 3). Degradation of the probe by nuclease present in the extract also occurred, if more than 2 µl of extract was used in the reactions (lanes 10-16 in Fig 2).

FIG. 2. Optimization of levels of transcription extract and non-specific DNA competitors for EMSA. Different amounts of crude cell extract (μl) as indicated at the top were used with 50,000 cpm (about 0.4 ng of DNA) each of a wild type 5S DNA (pFS415) probe in buffer 1 (see Materials and Methods). The DNA probe without extract is shown in lane 1. Lanes are grouped according to the amount of extract used. The amounts of non-specific competitor (poly (dI-dC)) used are indicated at the top of each lane. The arrows indicate the protein-DNA complexes designated I, II, and III.


Crude extract
Poly (dI-dC)
hg
III
II
I
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
free DNA
0.3
1.0
3.0
1.0 µl
0.0
0.3 *
1.0
3.0
1.5 µl
0.0
0.3
1.0
3.0
2.0 µl
0.0
0.3
1.0
3.0
4.0 µl

2.3.2. Specific protein-DNA interactions depend on an intact C box, and cannot be competed by any internal or upstream elements including the Ribo box in the absence of the C box. To determine which complexes (the three retarded bands in Fig 2) represented specific protein-DNA interactions, a series of competition experiments were carried out, using conditions standardized as above. Increasing amounts of competitor DNA, both non-specific and specific, were used to compete for formation of the DNA-protein complexes. Poly (dI-dC) and plasmid DNA (pUC18) were used as non-specific competitors respectively. When increasing amounts of poly (dI-dC) were included in the retardation reactions, the intensity of band I decreased, and finally disappeared completely with 6 μg of poly (dI-dC) (lane 4, Fig 3). However, bands II and III changed very little in intensities with increasing concentration of poly (dI-dC) but shifted in positions (compare lane 2 with lane 4, Fig 3). Similarly, bands II and III were relatively insensitive to competition by the plasmid DNA (compare lane 5 with 7, Fig 3). Band I was more sensitive to plasmid DNA than to poly (dI-dC) (compare lane 2 with 6, Fig 3) and was absent when more than 0.3 μg of plasmid DNA was used. This may be due to the fact that poly (dI-dC) is not a natural DNA. When the wild type 5S gene was used as a specific competitor, all the retarded bands disappeared at low concentrations of competitor (lanes 8-10, Fig 3), suggesting that bands II and III were due to specific interactions of proteins with the 5S DNA. With 5 mM EDTA included in the retardation reactions, the formation of

FIG. 3. Competition by non-specific and specific DNA competitors against the formation of protein-DNA complexes on the 5S gene. 1 μl crude cell extract was used with 50,000 cpm (about 0.4 ng of DNA) per reaction of wild type 5S gene (pFS415) in buffer 1. The DNA competitors are indicated at the top for each group. The amount of DNA competitor (μg) used is marked at the top of each lane.


Competitor
μg
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
free DNA
1.0
3.0
6.0
Poly (dI-dC)
0.3
1.0
3.0
Plasmid DNA
0.3
1.0
3.0
WT 5S plasmid
0.3
1.0
3.0
A + C box plasmid
0.3
1.0
3.0
Ribo box plasmid

band II and III was completely inhibited, while band I unaffected (data not shown), suggesting that different proteins were involved. It is known from other systems that the binding of 5S specific transcription factor TFIIIA to the 5S DNA requires zinc (reviewed by Geiduschek and Tocchini-Valentini, 1988) which can be chelated by EDTA. A plasmid which carried only the A and C boxes competed equally well as the plasmid with the wild type 5S gene (compare lanes 8-10 with 11-13 in Fig 3). This mutant does not carry a Ribo box and the 5' flanking sequences. In contrast, the Ribo box plus the 5' flanking sequences did not compete for the specific interactions even with large amounts of competitor DNA (compare lanes 14-16 with lanes 11-13, Fig 3), suggesting that the Ribo box alone is not capable of binding the proteins responsible for bands II and III.

To try to detect differences in protein binding between the wild type 5S gene and the Ribo box mutant, EMSAs were set up using the same conditions (buffer 2) used for *in vitro* transcription of the 5S gene (see Materials and Methods). Fig 4 compares side-by-side the binding patterns of the wild type 5S gene and the Ribo box substitution mutant (pFS126). Either ATP or all 4 NTPs (GTP, ATP, UTP, CTP) were used in combination with different amounts of calf thymus DNA as non-specific competitor. Band I and III were not observable under the conditions used. As shown in Fig 4, in no case were there any significant differences detected between the wild type 5S gene and the Ribo box mutant in the pattern or affinity of

FIG. 4. Comparison of a Ribo box mutant with the wild type 5S gene in formation of specific protein-DNA complexes under conditions optimized for *in vitro* transcription. 1 µl of extract was used with different amounts of calf thymus DNA (µg) as marked at the top of each group. The DNA probes (50,000 cpm each, about 0.4 ng) used were the wild type 5S (WT, pFS415) and Ribo box mutant (R-, pFS126). 5S transcription buffer (buffer 2) containing 4 mM of phosphoenol pyruvate was used in the absence of NTPs (NTP-), with 0.4 mM of ATP, or with 4 NTPs (GTP, ATP, UTP, & CTP, 0.4 mM each). Bands I and III were not observed under these conditions.

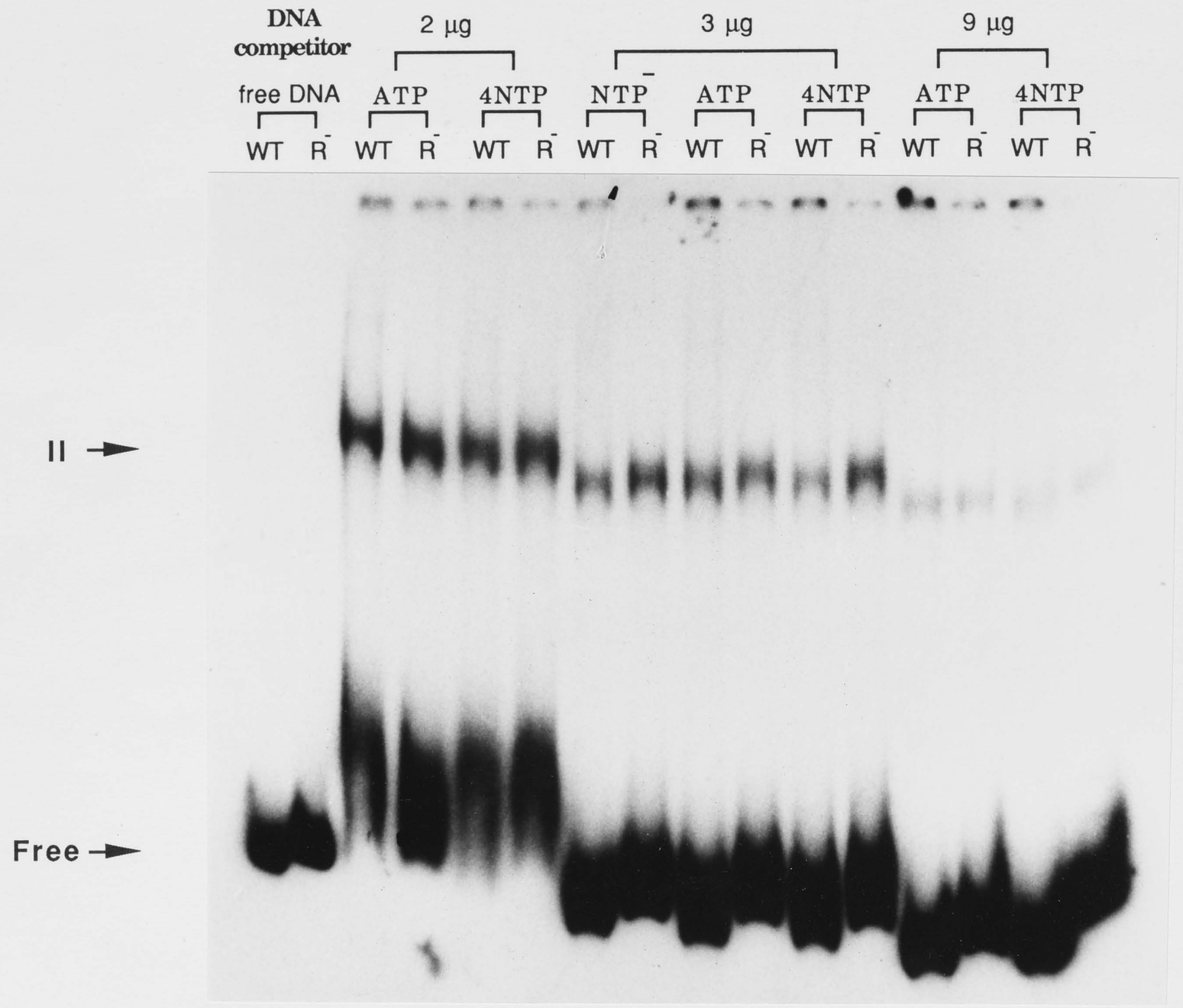
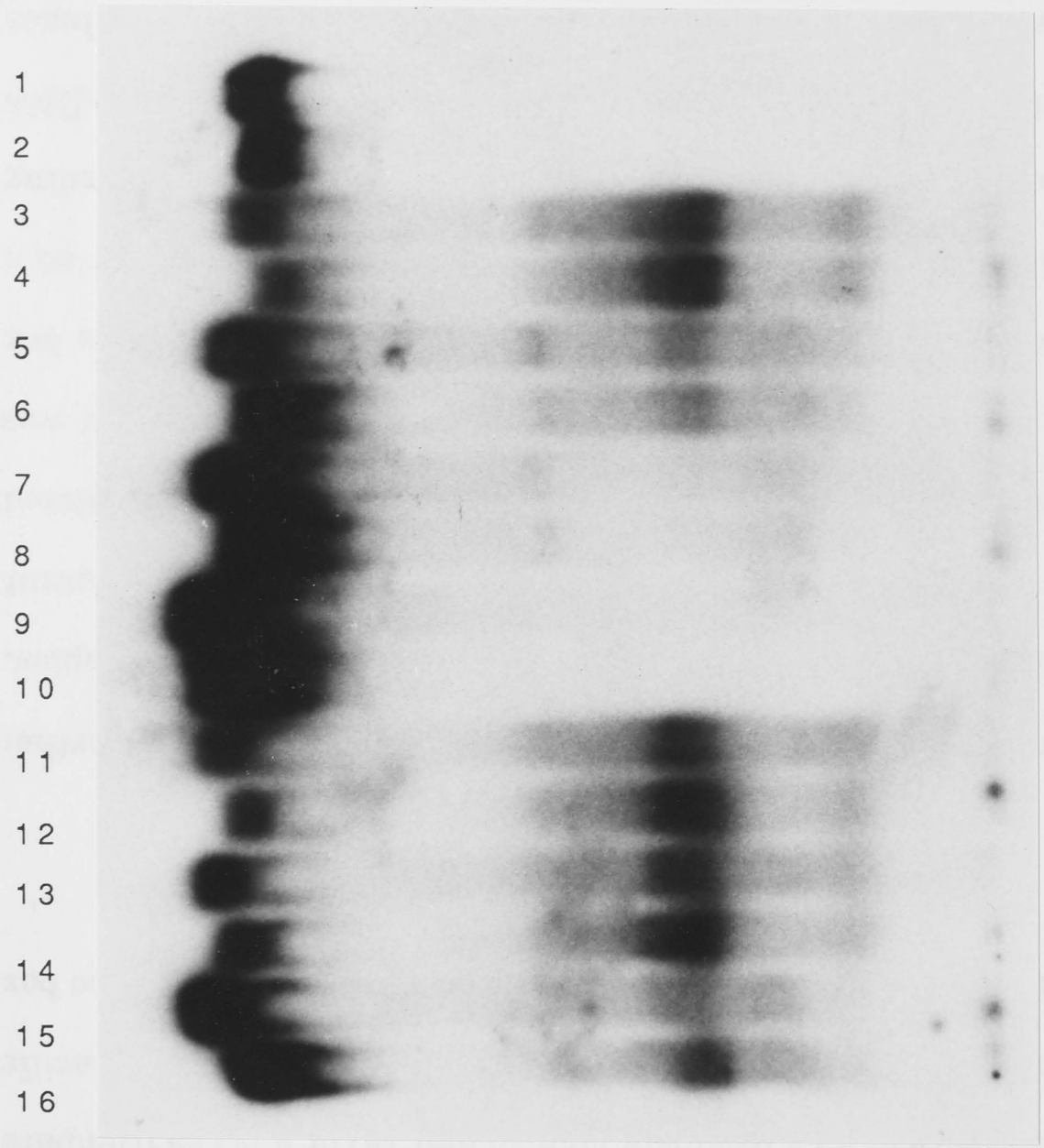

DNA competitor
2 µg
3 µg
9 µg
free DNA
ATP
4NTP
NTP⁻
ATP
4NTP
ATP
4NTP
WT R⁻ WT R⁻ WT R⁻ WT R⁻ WT R⁻ WT R⁻ WT R⁻ WT R⁻
II
Free
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 5. Competition by TATA and C box mutants against protein-DNA complexes formed on the Ribo box and A box mutants. Buffer 1 was used together with 1 μl of crude cell extract and 3 μg of poly (dI-dC). The DNA probes (50,000 cpm each, about 0.4 ng) were the Ribo box mutant (R-, pFS126) and the A box mutant (A-, pFS128). The DNA competitors are the TATA box mutant (TATA-, pFS210) and the C box mutant (C-, pFS332) as indicated at the top of the lanes. The amounts of 5S competitor DNAs used, ranging from 0.0 ng to 50.0 ng, are marked at the top of each lane.


DNA competitor
ng
free DNA
0.0
0.5
5.0
50.0
0.5
5.0
50.0
TATA
C
R
A
Free
II
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

binding (compare lanes 3 with 4, 5 with 6, 7 with 8, 9 with 10, 11 with 12, 13 with 14, and 15 with 16). In particular, although a high level of calf thymus DNA (9 μg) significantly competed for binding (lanes 13-16), no difference could be detected between the wild type and the Ribo box mutant either in the presence of ATP (compare lane 13 and 14) or 4 NTPs (compare lanes 15 with 16 in Fig 4). The results suggest that the specific protein-DNA interactions observed in this assay do not require the Ribo box sequence.

Specific competition experiments were carried out to examine further the role of the A and C boxes in forming a stable protein-DNA complex. Two specific competitors were used: a 5S TATA box deletion mutant (pFS210) in which the 5' flanking sequences upstream of -21 was deleted and a C box deletion mutant (pFS332) in which part of the C box was deleted (see Materials and Methods). The probes used were the Ribo box mutant described above and an A box substitution mutant which carried a large region of base substitution spanning the A box and the surrounding sequences (Tyler, 1987). The affinities and patterns of specific protein-DNA interactions were similar for the Ribo and A box mutants (compare lanes 3 with 4, Fig 5). Both mutants were sensitive to competition by the TATA box mutant (compare lanes 5 with 6, 7 with 8, 9 with 10), but not by the C box mutant (lanes 11-16, Fig 5). The results clearly demonstrated that the A box was not required for the specific protein-DNA interaction observed in

FIG. 6. DNase I footprinting. The DNA probes (50,000 cpm, about 0.4 ng) were the Ribo box mutant (pFS126) and the wild type 5S gene (pFS415). A+G: DNA sequence marker formed by A+G chemical sequencing of the probe; M: DNase I cleavage marker *(i. e.* no extract); C: calf thymus DNA as competitor; P: poly (dI-dC). The amounts of competitor DNAs used are marked at the top of the lanes. The vertical lines indicate the regions of the C, A, and Ribo boxes. The arrow marks the transcription initiation site (+1) of the 5S gene.

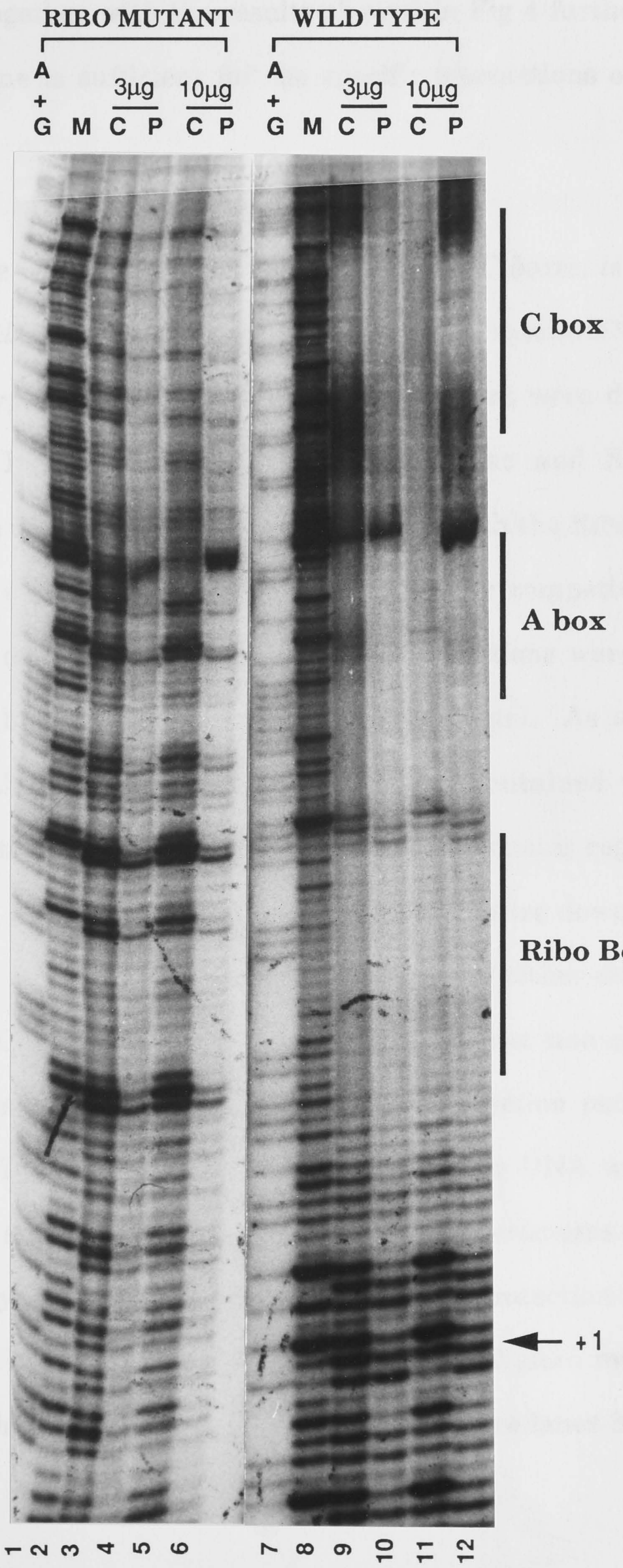

RIBO MUTANT
WILD TYPE
A + G
M
3µg
10µg
C
P
C box
A box
Ribo Box
+1
1 2 3 4 5 6 7 8 9 10 11 12

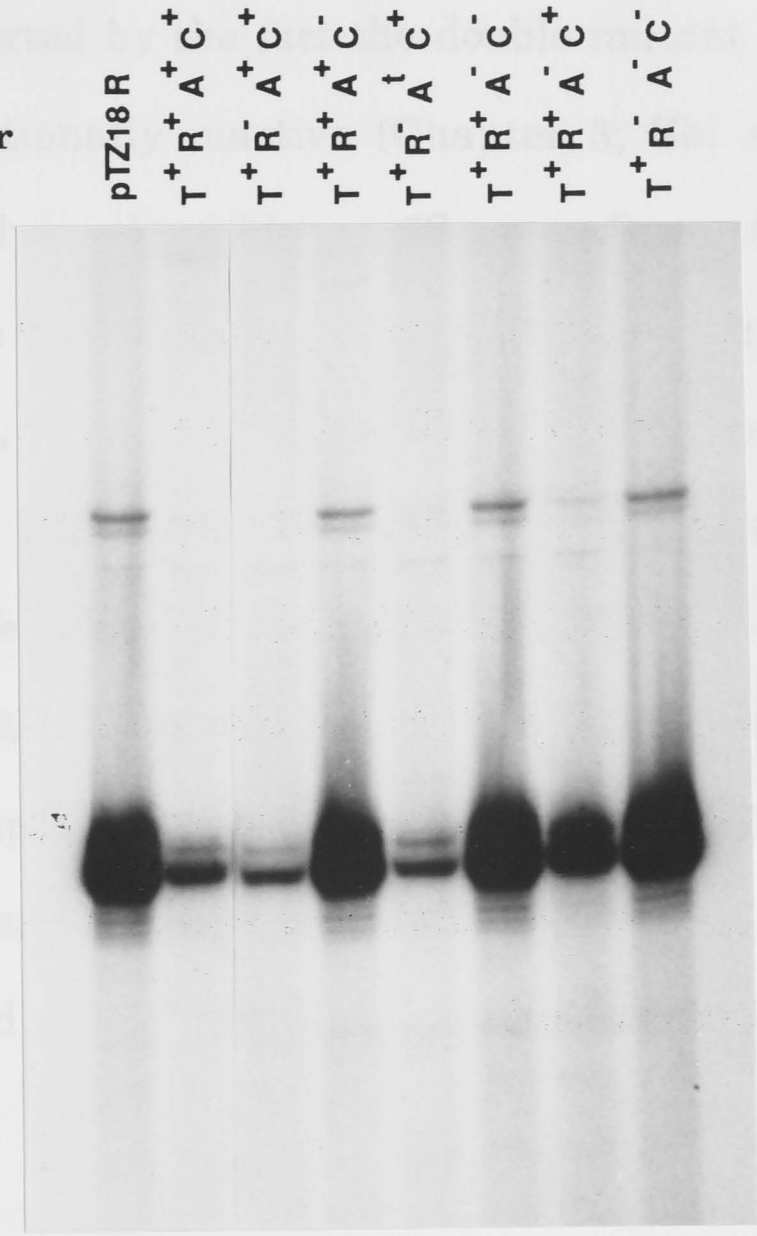
Fig 4. This together with the results showed in Fig 4 further suggest that the C box alone is sufficient for the specific interactions observed in the experiments.

2.3.3. The Ribo box, together with the A and C boxes, is protected from the DNase I cleavage by transcription factors. Protein-DNA interactions on the wild type 5S gene and the Ribo box mutant were delineated more precisely by DNase I footprinting assay (Galas and Schmitz, 1978). Binding to the wild type 5S gene was compared with the Ribo box mutant in the presence of different amount of non-specific competitor DNAs (calf thymus DNA or poly (dI-dC)). The binding conditions were adapted from EMSA using buffer 1 (see Materials and Methods). As shown in Fig 6 (lanes 9-12) the *N. crassa* whole cell extract contained protein factors which interacted specifically with the internal promoter region of the wild type 5S gene. The Ribo box, together with the entire downstream region, was protected from DNase I cleavage when 3 μg of either calf thymus DNA or poly (dI-dC) was used as a competitor against non-specific binding (compare lanes 9 & 10 with 8, Fig 5). The protection patterns were not altered significantly even when 10 μg competitor DNA was used (Fig 6 lanes 11, 12), suggesting that the protein-DNA interactions detected by the DNase I assay are specific. In contrast, partial protection was observable only in the C box region when the Ribo box substitution mutant was used as probe for the DNase I footprinting assay (compare lanes 3-6 with 2 in Fig

6). The exact border of the footprints could not be determined due to the activity of endogenous exonucleases present in the extract, which is indicated by the multiple banding of several strong bands present in the lanes with extract (lanes 9-12). This exonuclease activity was inhibited more effectively by the calf thymus DNA than by poly (dI-dC) (compare lane 9 with 10, 11 with 12, Fig 6). It should be noted that the DNase I footprinting assay was poorly reproducible due to interference by exonucleases and non-specific DNA binding proteins. Therefore the data in Fig 5 should be interpreted with caution. No footprint on the coding strand of the 5S gene could be detected (not shown).

2.3.4. The C box, but neither the Ribo box nor the A box, can compete for 5S transcription. Transcriptional competition experiments were carried out to verify the effect of the 5S mutations on the formation of transcription initiation complexes. The specific competitors used were 5S mutants carrying mutations in either the Ribo box, the A box, the C box, or a combination of mutations. The results demonstrated that transcription of the wild type 5S gene was inhibited 10 fold by the presence of a 19 fold excess of a wild type 5S gene which carried no termination site (compare lane a with b, Fig 7). A similar effect was observed when the competitor was either a 5S gene with a mutated Ribo box (lane c in Fig 7) or a 5S gene with a $tRNA^{Leu}$ A box (see Chapter 3) and a mutated Ribo box (lane e) (compare lane a with lanes c & e, Fig 7). The 5S mutant which carried a

FIG. 7. Competition against transcription of the wild type 5S gene by 5S mutants. 570 ng of competitor DNA was used for each transcription. The nature of the 5S mutants is marked at the top of each lane. The 5S mutant plasmids used were pFS14.1 (lane b); pFS415-19 (c); pFS37 (d); pFS415-1319 (e); pFS81 (f); pFS128 (g); pFS182 (h); also refer to Fig 1 for details. The transcription efficiencies relative to the wild type (lane a, 100%) are indicated at the bottom. See Materials and Methods section for detailed transcription conditions used.


DNA competitor
pTZ18R
T+R+A+C+
T+R-A+C+
T+R+A+C-
T+R-At C+
T+R+A-C-
T+R+A-C+
T+R-A-C-
100
11
8
81
9
69
23
84
a
b
c
d
e
f
g
h

large region of base substitution spanning the A box and the surrounding regions (lane g) also competed effectively for the 5S transcription, though not as strongly as the Ribo box mutants (compare lane g with c & e, Fig 7). The poorer competition might be caused by the mutation extending beyond the 3' end of the A box rather than by the mutation of the A box region itself, which is supported by the fact the double mutant (lane e in Fig 7) which was transcriptionally inactive (Chapter 3; Shi and Tyler, 1991) competed as effectively as the wild type 5S gene. By contrast, neither the TATA box alone (lane h), the Ribo box plus the TATA box (lane f), nor the combination of TATA, Ribo, and A boxes (lane d) could compete effectively for 5S transcription. The differences in transcription efficiencies in the presence of the different C box mutants (lanes d, f, and h) are not significant. The results suggest that the C box plays a dominant role in the formation of transcription initiation complexes on the 5S gene, and are consistent with the results from the EMSAs and the DNase I footprinting experiments described above.

2.4. DISCUSSION

Transcription by RNA polymerase III employs a wide variety of promoter elements among different types of genes (*i. e.* 5S rRNA gene, tRNA/VA RNA, U6/7SL snRNA genes) (reviewed by Geiduschek and Tocchini-Valentini, 1988). Although studies in *Xenopus* have served as a

paradigm for 5S gene transcription in eukaryotes, 5S genes from different organisms differ significantly in their promoter sequences both in the 5' flanking and the internal control regions. Transcription of 5S genes in *Xenopus* requires a tripartite internal control region (ICR) covering the A box, the intermediate box, and the C box. In addition, a 5' flanking sequence was reported recently to be important in modulating transcription efficiency and developmental regulation (Oei and Pieler, 1990; Jerzmanowski and Cole, 1990). Although the yeast 5S A and C boxes are conserved both in sequence and position relative to the *Xenopus* 5S gene (Braun *et al.*, 1989), recent mutational analysis showed that only the C box plus a 22 bp element spanning the +1 site are essential for transcription *in vitro* (Challice and Segall, 1989). In contrast, the 5S gene of *Drosophila melanogaster* contains four internal elements and an upstream element at -39 to -26. In *Neurospora crassa*, transcription of the 5S gene requires one additional internal element, the Ribo box, in addition to the A and C boxes as in *Xenopus* (Tyler, 1987) and a TATA box at -29.

The studies presented here investigated the contribution of each 5S gene promoter element of *N. crassa* to the formation of a transcription initiation complex. A crude whole cell extract that actively transcribes 5S genes *in vitro* was used as a source of transcription factors. Three different assays were employed in the studies: EMSA, DNase I footprinting, and transcriptional competition. It was shown by EMSA that

the crude extract contained protein factors that interacted with wild type 5S DNA, an interaction which resulted in protein-DNA complexes seen as retarded bands in an low-ionic-strength polyacrylamide gel. Two of the bands were resistant to competition by non-specific DNA competitors, such as poly (dI-dC) and plasmid DNA, but were sensitive to competition by wild type 5S DNA template, suggesting that the bands were caused by specific interaction of 5S transcription factors with the DNA template. Plasmid DNA was a stronger competitor for the non-specific protein-DNA complexes than poly (dI-dC) by a factor of 20.

Even though the TATA, Ribo and A boxes are essential for the efficient *in vitro* transcription of the 5S gene in *N. crassa*, their involvement in the formation of transcription initiation complex could not be detected by EMSA. No significant differences either in banding patterns or in the affinity of the specific interactions could be detected by EMSA between the wild type 5S gene and various 5S mutants, except the C box mutant, under a variety of assay conditions, including conditions used for *in vitro* transcription (Tyler, 1987). Similarly, all 5S mutants except the C box mutant competed as efficiently as the wild type 5S gene for the specific interactions revealed by the retarded bands.

The fact that the C box mutant failed either to form specific complexes (data not shown) or to compete for any specific retarded bands strongly

suggests that the specific interactions observed by EMSA were attributable only to the binding of protein(s) to the C box region. This protein is most likely to be TFIIIA, based on studies of 5S transcription factors in other systems (Geiduschek and Tocchini-Valentini, 1988). The fact that the specific retarded bands, but not the non-specific bands, were sensitive to EDTA treatment supports the idea, because the binding of TFIIIA in other systems requires zinc ions (Geiduschek and Tocchini-Valentini, 1988). This hypothesis also is supported by the results from the transcription competition experiments. Various 5S mutants, including those used in EMSA, were used as specific competitors of transcription of the wild type 5S gene. Mutations either in the TATA, Ribo, A boxes, or combinations of them had no significant effect on competition with the wild type 5S gene, while the mutations within the C box region abolished competition. Therefore in *N. crassa* a TFIIIA-like factor is probably the most important transcription factor for the formation of initiation complexes on the 5S gene. In the absence of the factor (or the C box), no transcription factors appear to be committed to the DNA template. This is consistent with the findings in other systems (Sakonju *et al.*, 1981; Geiduschek and Tocchini-Valentini, 1988).

The involvement of the internal control elements in formation of the initiation complex in *N. crassa* was examined here by DNase I footprinting using a whole cell extract. In contrast to the results from the EMSAs, the

contributions of the Ribo and A box to the formation of the initiation complexes could be detected in some experiments. Both the Ribo box and the entire downstream region could be protected from DNase I cleavage in the wild type 5S gene. By comparison, only partial protection could be detected within the C box region when the probe used was a Ribo box mutant. The footprints are coincident with the regions defined as important for *in vitro* transcription of the 5S gene (Tyler, 1987). However, it should be emphasized that the DNase I footprinting assays using crude cell extracts were difficult and poorly reproducible, due to the presence of abundant non-specific DNA binding proteins and exonucleases. Braun *et al.*, (1989) also reported that numerous attempts to footprint the yeast 5S gene with crude extracts proved fruitless.

It is intriguing to note that the differences between the wild type and the mutant 5S gene shown by the DNase I assay were not detected by EMSA. One possible explanation is that the conditions used for DNase I footprinting were more gentle than those used for EMSA, which involved a 3-4 hour electrophoresis through a polyacrylamide gel. A similar problem has been reported in studies of the human transcription factor TFIID. Though TFIID has been clearly demonstrated by DNase I footprinting assays to bind the TATA sequence, the binding could not be detected by EMSA (Sawadogo and Roeder, 1985; Nakajima *et al.*, 1988; Horikoshi *et al.*, 1990; Pugh and Tjian, 1990). This is due to TFIID's high dissociation

constant. In yeast, the band formed by the TFIIIA.5SDNA complex is further retarded with the commitment of TFIIIC to the complex (Challice and Segall, 1989). However, no differences in band patterns were detected in *N. crassa* between the wild type 5S gene and a variety of 5S mutants carrying an intact C box. This could be caused by weak binding of TFIIIC and TFIIIB-like factors in *N. crassa*, resulting in complexes which were easily dissociated under conditions used for the EMSAs. The current data also favor the notion that the critical contacting points of *N. crassa* TFIIIA-like factor are mainly located in the C box region. There may also be weak contact points located in the region between the Ribo and C boxes, as mutations within the region reduced the ability to compete with the wild type 5S gene for transcription *in vitro* (compare lane b with g in Fig 7).

TFIIIC alone has been reported from studies on the *Xenopus* and yeast 5S genes to have very low affinity for the DNA template (Challice and Segall, 1989; Pieler *et al*., 1987). In yeast, weak binding of TFIIIC to the 5S template has been detected by EMSA. It is likely the binding is not sequence specific because it is easily competed by non-specific DNA competitor (Challice and Segall, 1989). Similarly, *N. crassa* TFIIIC is unlikely to have sequence specific recognition of the template, because in the absence the C box, no other internal elements alone or in combination could compete for 5S transcription. Consequently, the hypothesis that only the interaction of TFIIIA with the C box region was detected from the

EMSAs could be tested using a *N. crassa* tRNA gene as competitor for the common transcription factors TFIIIC and TFIIIB. It could also be verified using purified or partially purified factors, which will identify which factor is responsible for the footprint within the Ribo box and A box region.

Specific efforts have been made throughout these studies to identify a transcription factor which might recognise the Ribo box, due to the potential role the Ribo box may play in coordinating transcription of ribosomal genes (Tyler, 1987; 1990; Tyler and Harrison, 1990). The Ribo box is essential for *in vitro* transcription of the 5S gene, and a 2 bp substitution within the region reduces the transcription 4-fold (Tyler, 1987). However, no significant differences could be detected between the wild type 5S gene and the Ribo box mutants either in banding patterns or in the affinity of the retarded bands under a variety of binding conditions. In the absence of the C box, the Ribo box could not compete for specific retardation or for transcription of the 5S gene *in vitro* either alone or combined with 5' flanking sequences or the A box. These results argue strongly against the existence of a transcription factor which interacts with the Ribo box independently. Yet it is still possible that a secondary transcription factor, like TFIIIC, exists which recognizes a part of the Ribo box sequence but whose commitment to the transcription initiation complex depends on the prior-association of other transcription factors with the DNA template, such as TFIIIA. The fact that the Ribo box can be protected by a transcription complex from DNase cleavage favors such a possibility.

CHAPTER 3

ALL INTERNAL PROMOTER ELEMENTS OF 5S rRNA AND tRNA GENES, INCLUDING THE A BOXES, ARE FUNCTIONALLY GENE–SPECIFIC

3.1. INTRODUCTION

The initiation of transcription by RNA polymerase III on 5S rRNA and tRNA genes shares some common features. Both genes have internal promoter sequences and require transcription initiation factors TFIIIC and TFIIIB (see Geiduschek and Tocchini-Valentini, 1988; Palmer and William, 1990 for review). Yet the way in which these common transcription factors interact must be different, because the critical promoter elements, the C box of 5S genes and the B box of tRNA genes, are gene-specific. The C box is the binding site for the 5S–specific transcription factor TFIIIA. Binding of TFIIIA is a prerequisite for subsequent binding of TFIIIC, TFIIIB, and RNA polymerase III. In tRNA genes TFIIIC binds directly to the B box and does not require the participation of TFIIIA (Geiduschek and Tocchini-Valentini, 1988; Sharp *et al*., 1985). Both 5S and tRNA genes require a second element, called the

A box, which has been considered to be functionally interchangeable between the two genes (Ciliberto *et al.*, 1983; Pieler *et al.*, 1987). The role of the A box is not well understood. In *Xenopus* 5S genes, TFIIIA alone contacts part of the A box (Fairall *et al.*, 1986; Pieler *et al.*, 1987; Sakonju *et al.*, 1981). Certain A box mutations interfere with the binding of TFIIIC to the TFIIIA•5S DNA complex (Pieler *et al.*, 1987), but no binding of TFIIIC to the A box can be detected by DNaseI footprinting (Carey *et al.*, 1986; Fradkin *et al.*, 1989). In yeast, the A box is protected from DNase I cleavage only in the presence of all three factors TFIIIA, TFIIIC and TFIIIB (Kassavetis *et al.*, 1990). In contrast, in tRNA genes, TFIIIC alone clearly contacts the A box following binding to the B box (Baker *et al.*, 1986; Carey *et al.*, 1986; Kassavetis *et al.*, 1990). Binding of TFIIIC is followed by the binding of TFIIIB and RNA polymerase III. It is not known how TFIIIC participates in the different types of initiation complexes found on the 5S and tRNA genes. Additional 5S-specific elements have been reported in *Drosophila* (Sharp and Garcia, 1988) and *Neurospora crassa* (Tyler, 1987).

In addition to the internal sequences, the -10 region and upstream flanking sequences of the *Xenopus* 5S gene (Oei and Pieler, 1990) and the -14 to +8 region of the yeast 5S gene (Challice and Segall, 1989) are also important in modulating transcription efficiency *in vitro*. In yeast, 43 bp of the 5' flanking sequences were protected from DNase I cleavage during

transcription initiation *in vitro,* as well as the internal control region (ICR) (Braun *et al*., 1989). In other organisms, such as *Drosophila* (Sharp and Garcia, 1988), silkworm (*B. mori*) (Morton and Sprague, 1984), and *Neurospora crassa* (Tyler, 1987), 5' flanking sequences including TATA-like sequences are also important. Some RNA polymerase III transcribed genes, such as the human U6 snRNA, require only 5' flanking sequences (Das *et al*., 1988; Murphy *et al*., 1987).

Transcription of 5S genes in *N. crassa* requires A and C boxes similar to the *Xenopus* ICR, plus a TATA sequence at -29 which directs the startpoint of transcription. It also requires an extra internal element at +18 to +34, the Ribo box (Tyler, 1987). The Ribo box is essential for transcription of 5S genes *in vitro*, but is not found in *N. crassa* tRNA genes. A Ribo box is also required for efficient transcription *in vitro* of 40S rRNA genes by RNA polymerase I (Tyler, 1990). Furthermore, three copies of the Ribo box sequence are present in the 5' flanking sequence of the ribosomal protein gene, *crp-2* (Tyler and Harrison, 1990). Therefore the Ribo box may coordinate the transcription of ribosomal component genes in *N. crassa*. The *N. crassa* 5S A box has homology to the consensus A box sequence of eukaryotic tRNA genes (Geiduschek and Tocchini-Valentini, 1988), but with mismatches at two key positions. The potential regulatory role of the Ribo box and its absence from tRNA genes made it possible to investigate the influence, if any, of the Ribo box on tRNA transcription and

its interaction with the different 5S and tRNA A box sequences. It was found that the 5S and tRNA A boxes were not functionally identical and function well only in their native context. Furthermore, the Ribo box was specific to the 5S gene and was not required for tRNA transcription.

3.2. MATERIALS AND METHODS

3.2.1. Plasmid and DNA manipulation. The 5S and tRNA genes used in these studies were the 5S gene α52 (Selker *et al.*, 1981), and a cloned $tRNA^{Leu}$ gene (Huiet *et al.*, 1984). Two major families of transcription templates were used. The first was the wild type α52 gene located on a 390 bp *Sal-Pst* fragment inserted into pTZ18R (Pharmacia) and mutant 5S genes derived from it (Fig 1B, *a-d*). The second was a hybrid 5S-tRNA gene constructed by ligating the 5' part of the α52 5S gene to the 3' part of the $tRNA^{Leu}$ gene in pTZ18R, and mutants derived from the hybrid gene (Fig 1B, *e-h*). The 5S portion of the hybrid extends from the *SalI* site at -230 to the *SpeI* site at +72, and includes the TATA, Ribo, and A boxes. The tRNA portion of the hybrid extends from the *HpaI* site at +49 to a *ClaI* site 200 bp beyond the 3' end of the gene, and includes the B box and the transcription terminator (Fig 1B, line *e*). All plasmid DNA was purified by CsCl gradient centrifugation until no RNA could be detected. Oligonucleotide-directed mutagenesis was carried out according to Nisbet

and Beilharz (1985) with uridine enrichment (Kunkel, 1985). Single stranded DNA used for mutagenesis was prepared by means of the f1 phage origin of replication in pTZ18R, by super-infecting the host cells with M13K07 (Vieira and Messing, 1987). All mutants were sequenced either by dideoxy sequencing with alkali-denatured double stranded plasmid DNA or by chemical cleavage (Maxam and Gilbert, 1980) with a slight modification (Shi and Tyler, 1989).

3.2.2. In vitro transcription assay. Transcription extracts were prepared from *N. crassa* strain 105C by the methods of Tyler and Giles (1984). The standard 25 µl transcription reaction (Tyler, 1987) contained 4 µl of extract, 10 mM KHepes (pH 7.9), 5 mM K_2 ethylene glycol-bis (b-aminoethylether) N,N,N',N' tetraacetate (EGTA), 103 mM K-acetate, 10 mM MgOAc, 2.5% glycerol, 2.5 mM dithiothreitol, 400 µM each ATP, CTP and GTP, 50 µM UTP, 0.1-10 µCi [α-^{32}P] UTP, 4 mM K-phosphoenolpyruvate, 3.9 x 10^{-10} M (about 30 ng) circular template DNA and 570 ng plasmid carrier DNA. Incubation was for 20 min at 30 ^{o}C. The carrier DNAs were either pTZ18R (for normal transcription) or a specific plasmid competitor DNA (for competition experiments). Competition experiments (unpublished) showed that 4 µl of transcription extract contained about 11 fmol C-box binding activity (presumably TFIIIA), sufficient to saturate about 30 ng of a 5S plasmid, plus about 16 fmol B-box

binding activity (presumably TFIIIC), sufficient to saturate about 45 ng of a $tRNA^{Leu}$ plasmid. The concentrations of all template and carrier DNAs were measured at least twice by fluorometer using bisbenzamide dye. In the case of competition experiments, the competitor DNA was premixed with all other components including the transcription extract on ice for 5-10 min before addition of template DNA. For simple chase experiments, the reaction with [α-^{32}P]-UTP was allowed to proceed for 20 min, then 400 μM unlabelled UTP was added and the reaction extended for 10 more minutes. For pulse transcription reactions, template DNA was incubated with unlabelled UTP (50 μM) for 20 min at 30 ^{o}C, then 10 μCi [α-^{32}P]-UTP was added for a further 30 seconds. All reactions were stopped by addition of 100 μl of 100 mM sodium acetate (pH 5), 10 mM EDTA, 4% SDS. RNA transcripts were purified by extraction with 150 μl of phenol/chloroform/isoamyl alcohol (50:50:2 v/v) and ethanol precipitation, and were fractionated by electrophoresis on 7 M urea/8% polyacrylamide gels. Transcription efficiencies were measured by scanning the dried gels using an AMBIS radioanalytic imaging system (San Diego, CA). All the data presented here are the average of at least two independent experiments, with a range of less than 10%.

3.3. RESULTS

3.3.1. The tRNA^{Leu} A box functions poorly in a 5S gene. The A box of the *N. crassa* α52 5S rRNA gene differs at 5 base positions from that of the *N. crassa* tRNA^{Leu} gene and at two positions from the eukaryotic tRNA A box consensus (Fig 1*A*). The 5S A box sequence was changed by oligonucleotide mutagenesis to match the tRNA^{Leu} A box, except for a single base pair insertion allowed by the consensus (*415-13* in Fig 1*A*) (The term "altered A box" will be used to refer to the 415-13 A box sequence). Fig 1*B* shows the structure of the wild type 5S gene (*line a*), and the 5S gene with the altered A box (*line b*). Transcription of the 5S gene with the altered A box was reduced 7-fold (Fig 2, *lane b*) compared to the wild type (*lane a*), indicating that the tRNA A box sequence functioned poorly in the 5S gene. The A box alteration was also introduced into a 5S gene containing a 12 bp Ribo box substitution. The sequences of the wild type and mutant Ribo boxes are shown in Fig 1*A*. The structures of the 5S genes with the mutated Ribo box (*line d*), and with the mutated Ribo box and altered A box (*line c*) are shown in Fig 1*B*. The Ribo box mutation reduced transcription of the wild type gene 33-fold (Fig 2, *lane d*) and abolished the remaining transcription of the 5S gene containing the tRNA A box (*lane c*). Therefore the Ribo box is required for 5S transcription even in the presence of a tRNA A box sequence. The altered A box sequence in

the 5S gene did not affect the length of transcripts (compare *lane a* with *b*).

3.3.2. Transcription of 5S-tRNA^{Leu} hybrids depends on the A box but not the Ribo box. The interaction of the Ribo box and A box sequences with other internal elements was investigated using 5S-tRNA^{Leu} hybrid genes. The structures of these hybrid genes are shown in Fig 1*B line e* through *h*. The structure of the "wild-type" 5S-tRNA^{Leu} gene is shown in line *e* in Fig 1*B*. The 5' region of this hybrid gene carries a fragment of the wild type α52 5S gene from -230 to +72, including the Ribo box and the A box. The 3' portion of the hybrid consists of a fragment of the tRNA^{Leu} gene downstream from +49, including the B box and the transcription terminator. The distance between the 5S A box and tRNA^{Leu} B box in the hybrid is 58 bp compared with 67 bp between A and B boxes in the wild type tRNA^{Leu} gene. Transcription of the "wild type" 5S-tRNA^{Leu} hybrid produced a major transcript of 156 nt and minor transcripts of 144 and 138 nt (Fig 2, *lane e*). The transcription level (including all transcripts) was 7% and 8% respectively of the wild type 5S (*lane a*) and tRNA^{Leu} genes (*lane i*). RNase mapping of the transcripts of the hybrid indicated that the major transcript resulted from initiation at the 5S transcription initiation site (data not shown). Pulse–transcription experiments in which

FIG. 1. Structures of the transcription templates. *A*. Sequence comparisons between the 5S and tRNA^{Leu} A boxes, and sequence of the mutant Ribo box. 415-13 is a 5S A box mutant in which the sequence has been altered to match the tRNA consensus. N: any nucleotide. R: purine. Y: pyrimidine. *B*. Structures of the 5S and 5S-tRNA^{Leu} hybrid transcription templates showing the A box and Ribo box mutations. A^{5S} and A^{t} indicate 5S or tRNA-like A boxes (*A*). R+ and R- indicate wild-type or mutant Ribo boxes (*A*). Templates *a* & *i* are the wild-type 5S rRNA and tRNA^{Leu} genes, respectively; *b-d*, 5S mutants; *e-h*, 5S-tRNA^{Leu} hybrids, including mutants. T, B and C indicate the TATA, B and C boxes. +1 indicates the start points of 5S and tRNA transcription. Shaded Ribo and A boxes indicate mutant sequences. Positions of the 5S elements are from Tyler (1987). Positions of the tRNA elements are by homology to tRNA genes of other organisms.

A

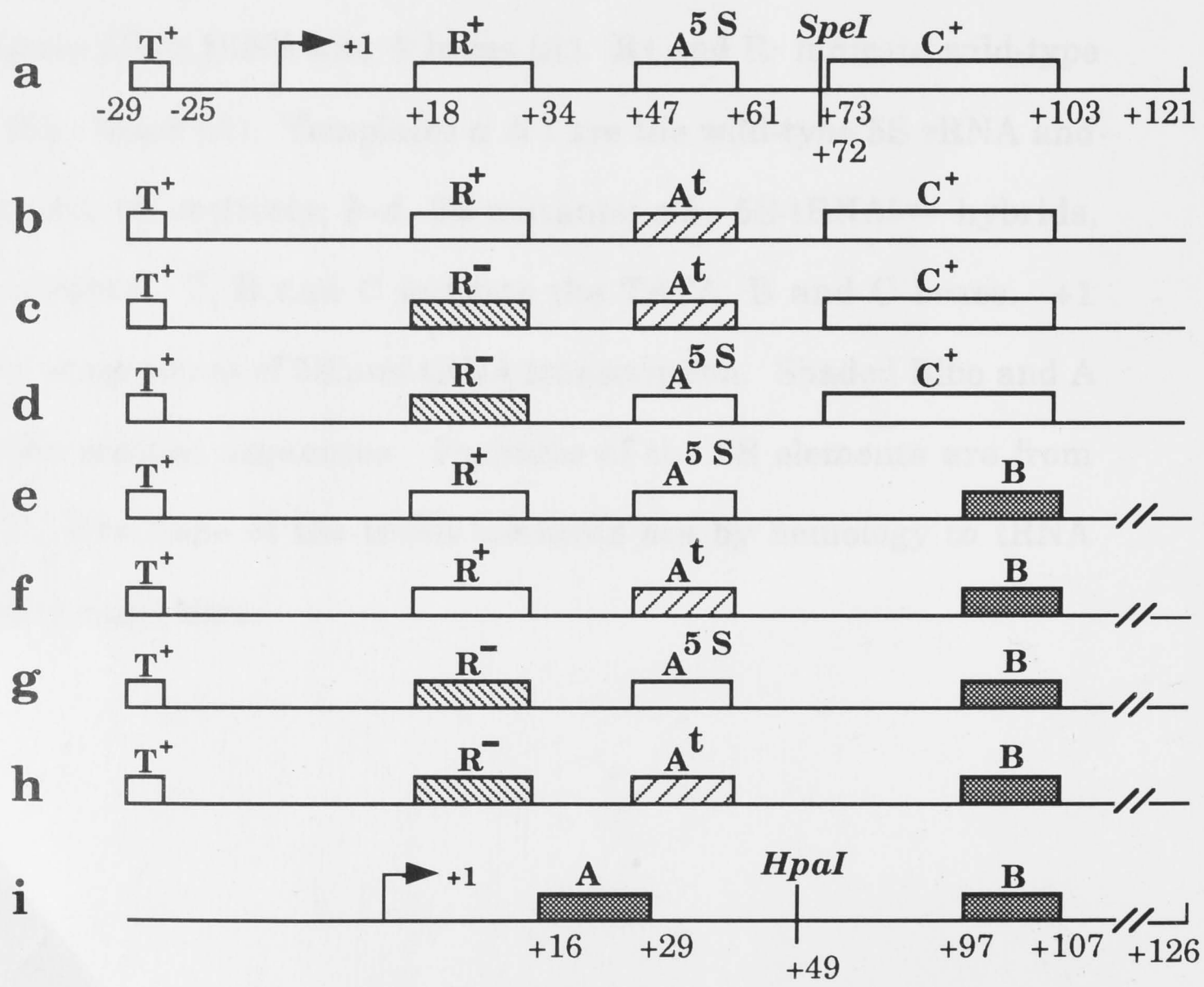
A BOX

tRNA consensus	5' TRGCNNAGYnGG 3'
$tRNA^{Leu}$	5' AGATGGCCGAGC GGT 3'
	5' CCATAGATAAGCCAGTG 3'
$5S(A^{5S})$	
$415\text{-}13\ (A^{t})$	5' CCATgGccgAGCCgGTG 3'

RIBO BOX

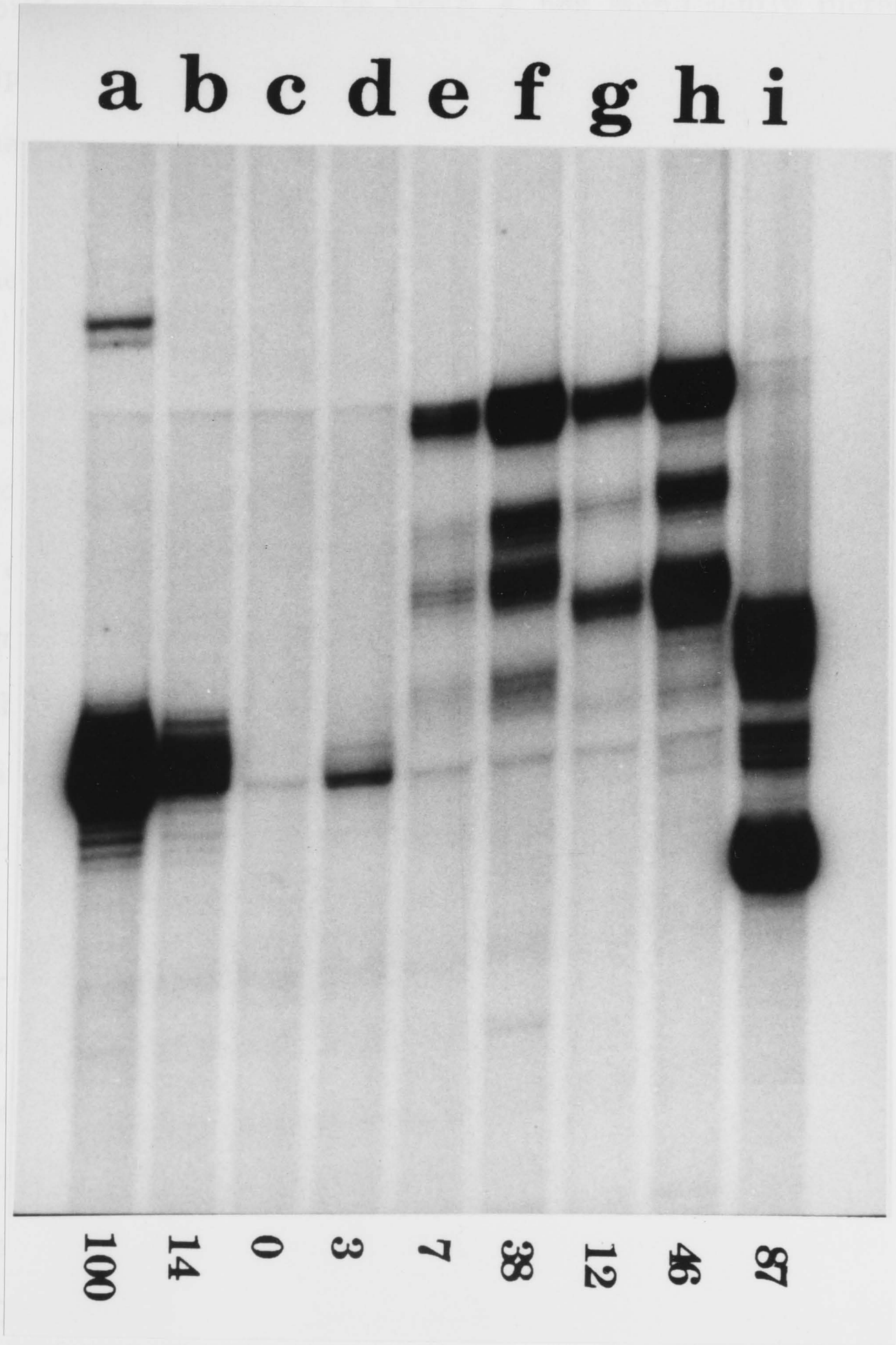
Ribo box (R^{+})	5' CTGGAAAACTCGGGATC 3'
Ribo box mutant (R^{-})	5' CTGctgtctagactgTC 3'

B



transcription was carried out in the presence of labelled UTP for only 30 seconds, and chase experiments in which labelled RNA transcripts were incubated for 10 minutes in presence of unlabelled UTP, indicated that the minor transcripts were not due to processing of the major transcript. Rather they reflected multiple initiation sites. The chase experiments also showed that both the major and minor transcripts from the hybrids were as stable as the wild type 5S transcripts (data not shown). The effect of different A box sequences on transcription of the 5S-tRNA^{Leu} hybrid gene was investigated by changing the 5S A box sequence to the 415-13 A box sequence (Fig 1*A*; Fig 1*B*, *line f*) as described in the previous section. As shown in Fig 2, the hybrid containing the tRNA A box (Fig 2, *lane f*) was transcribed five-fold more strongly than the hybrid containing the 5S A box (*lane e*). To test whether the Ribo box was required for transcription of the hybrid genes, the 12 bp Ribo box substitution mutation was introduced either alone (Fig 1*B*, *line g*) or in combination with the tRNA A box sequence (Fig 1*B*, *line h*). As shown in Fig 2, the transcription level of a hybrid with a mutant Ribo box was not significantly different from that of a hybrid with a wild type Ribo box either in the presence of a 5S A box (*lanes g* versus *e*) or of a tRNA A box (*lanes h* versus *f*). In fact, the Ribo box mutation slightly increased the transcription level and also altered the initiation site of some minor transcripts (compare *lane e* with *g*, *lane f* with *h*).

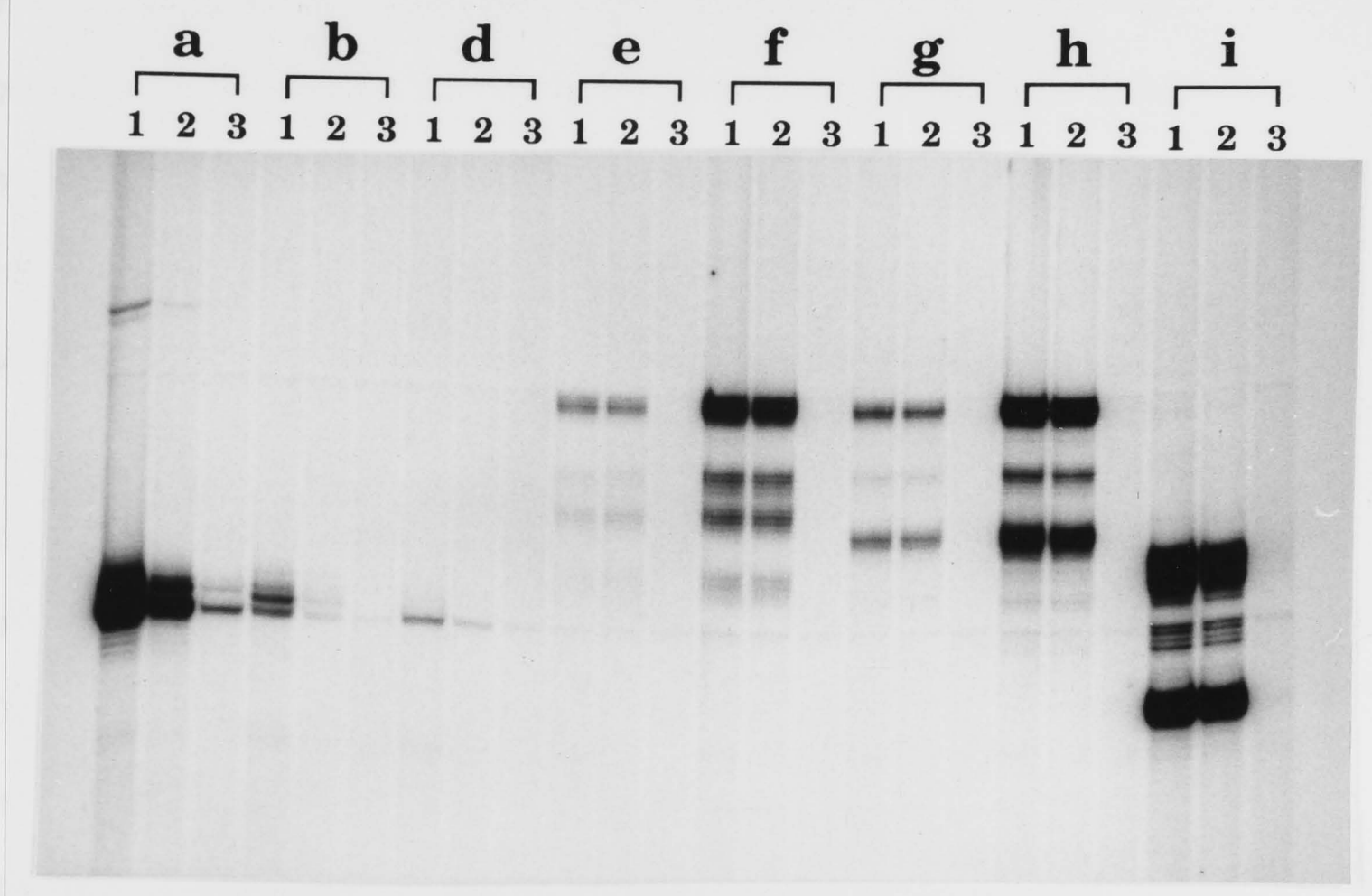
FIG. 2. Transcription of the 5S, 5S-tRNA^{Leu} hybrid and tRNA^{Leu} genes, and mutants of them. Lettering of the lanes corresponds to the templates shown in Fig 1B. Transcription efficiencies at the bottom of the figure are relative to the wild-type 5S gene (lane *a*) and were corrected for the length and uridine content of the transcripts. Transcriptions were for 20 min at 30 °C as described in the Materials & methods. The multiple tRNA transcripts (*i*) reflect processing of the primary transcripts while the multiple 5S-tRNA hybrid transcripts (*e-h*) reflect multiple initiation sites (see text)


a b c d e f g h i
100
14
0
3
7
38
12
46
87

In summary, the Ribo box was not required at all for transcription of the hybrid genes, whereas the tRNA A box significantly increased transcription compared to the 5S A box. Therefore, the tRNA A box functioned better than the 5S A box in a tRNA-like transcription unit (the hybrid), whereas, as shown in the previous section, the tRNA A box functioned worse than the 5S A box when placed in a 5S gene.

3.3.3. The 5S C box competes only for 5S transcription, while the tRNA B box competes for both 5S and tRNA transcription. The gene-specific nature of the 5S and tRNA internal elements made it necessary to investigate whether any transcription factors were common to the genes, as in other systems. Transcription competition experiments were carried out against the wild-type and mutant 5S genes, the 5S-tRNA hybrids, and the $tRNA^{Leu}$ gene. The 5S C box alone (which presumably binds TFIIIA) and the $tRNA^{Leu}$ B box alone (which presumably binds TFIIIC) were used as gene-specific competitors. The levels of competitor DNA used were in 10-20 fold molar excess of the relevant factors. The competition results are presented in Fig 3 with the transcription templates indicated as in Fig 1*B*. Transcription of the wild type and mutant 5S genes (Fig 3, *groups a*, *b*, and *d*), were all inhibited by prior incubation of the transcription extracts with C box DNA (*lane 2* in each group) compared with transcription of these genes in the presence of the same amount of pTZ18R DNA (*lane 1* in each group). In particular, transcription of the 5S gene with the tRNA A box

FIG. 3. Competition of the C box and the B box against the transcription of the 5S, the 5S-tRNA^{Leu} hybrid and tRNA genes, and their derivatives. Lettering indicates the transcription templates as diagrammed in Fig 1B. The numbers indicate the competitor DNA: 1, pTZ18R; 2, the C box; 3, the B box. 570 ng (185 fmol) of the competitor plasmid was preincubated with the extract for 5-10 min on ice prior to addition of ~30 ng (10 fmol) of transcription template. The temperature was then raised to 30 ^{o}C for 20 min.


a b d e f g h i
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

remained sensitive to C box competition (compare *lanes1* and *2* in *group b*). In contrast, the C box competition did not affect the transcription of the 5S-tRNA hybrids (compare *lanes 1* and *2* in *groups e-h*), nor did it affect transcription of the $tRNA^{Leu}$ gene itself (compare *lanes1* and *2* in *group i*). Competition with B box DNA totally abolished transcription not only of the hybrids and the $tRNA^{Leu}$ gene, which contain B boxes, (compare *lane 1* with *3* in *groups e-i)*, but also of all the 5S genes (compare *lane 1* with *3* in *groups a-d*). These results indicate that the 5S and tRNA genes share at least one transcription factor.

3.4. DISCUSSION

The internal control region of *Neurospora crassa* 5S genes consists of an A and C box as in *Xenopus* plus an additional element, the Ribo box. The Ribo box also is found in the 40S rRNA genes and the ribosomal protein gene, *crp-2*. *In vitro* transcription studies have shown that the Ribo box is absolutely required for efficient transcription of both the 5S and the 40S genes (Tyler, 1987; 1990). However, the Ribo box is absent from *N. crassa* tRNA genes. The *N. crassa* 5S A box has homology to the consensus A box sequence of eukaryotic tRNA genes (Geiduschek and Tocchini-Valentini, 1988). However, it has mismatches at two key positions, in contrast to *Xenopus* where the A box of the somatic 5S genes matches the tRNA consensus at all positions (Ciliberto *et al.*, 1983). The

difference between the 5S and tRNA A box sequences in *N. crassa* has made it possible to critically test whether the two A boxes are functionally interchangeable. Furthermore, it could be tested whether the difference between the A box sequences related to the function of the Ribo box. For example, perhaps the A box of the 5S gene was weak in comparison to that of the tRNA gene, and therefore required the Ribo box in order to support transcription initiation. It was tested whether the replacement of the 5S A box with a tRNA A box sequence would stimulate transcription of the 5S gene, and whether it would remove the requirement for the Ribo box. In fact, it was found that the altered 5S gene containing the tRNA A box was transcribed with low efficiency. Moreover, this altered 5S gene still depended on the Ribo box for transcription, and transcription was initiated from the normal site.

A 5S-tRNA hybrid was constructed to test the functions of the Ribo box and 5S A box in a tRNA-like transcription unit. The structure of the 5S-tRNA hybrid differs from the natural 5S genes in that the C box (the presumed binding site for TFIIIA) has been replaced by the B box of a $tRNA^{Leu}$ gene (the presumed binding site for TFIIIC). The spacing between the A and B boxes is 58 bp in the hybrid compared with 67 bp in the wild type $tRNA^{Leu}$. It is unlikely, though not impossible, that the 9 bp change of spacing affected the transcription of the hybrid, since transcription of a yeast $tRNA_3^{Leu}$ gene was insensitive to the spacing and

helical orientation of the A and B boxes (Baker *et al.*, 1987; Fabrizio *et al.*, 1984). In fact, the transcription level of the most efficient hybrid (*lane h*, Fig 2) approaches that of the wild type $tRNA^{Leu}$ gene (*lane i*, Fig 2). The transcriptional requirements of the hybrid differed from those of the 5S genes and were tRNA-like. In particular, competition experiments showed that, like the $tRNA^{Leu}$ gene, transcription of the hybrid did not require the 5S-specific C box binding factor (presumably TFIIIA). Conversion of the A box of the hybrid to that of a $tRNA^{Leu}$ gene improved transcription 4-5 fold, without changing the start points of transcription. This result confirmed the tRNA-like nature of the hybrid. More importantly, it demonstrated that the 5S A box sequence is in fact a poor tRNA A box, and contrasted with the finding that the tRNA A box sequence functions poorly in the 5S gene. The fact that both the 5S and tRNA A box sequences function poorly within a heterologous context strongly suggests that they are not functionally interchangeable, as previously assumed. The second major difference between the 5S and hybrid genes was that transcription of the hybrids was completely independent of the Ribo box, even in the presence of the 5S A box sequence. A 12 bp Ribo box mutation reduced 5S transcription 33 fold, but the same mutation actually increased hybrid transcription slightly. Two minor transcripts initiated within the Ribo box of the hybrid had altered start points in the mutant; in *N. crassa* (Tyler, 1987) and in *Xenopus* (Sakonju *et al.*, 1980) it has been shown previously that the DNA sequence at the initiation site influences selection

of the site. The strong requirement for the Ribo box in the natural 5S gene but not in the 5S-tRNA hybrid, in addition to the fact that the A box sequences are gene-specific, means that all three of the internal promoter elements of the *N. crassa* 5S gene (Ribo box, A box and C box) are functionally different from those of the tRNA gene.

The gene-specific nature of the internal elements of the *N. crassa* 5S and tRNA genes led to investigation on whether any common transcription factors were shared between the genes. In other systems, competition experiments and purification of individual factors have shown that TFIIIB and TFIIIC are required for both 5S and tRNA transcription, though it has not been ruled out that different forms or different subunits of the two factors might be used for the two genes (Kassavetis *et al.*, 1990; Lasser *et al.*, 1983; Segall, 1986). Competition experiments were conducted on 5S and tRNA genes and derivatives of them using C box and B box sequences as specific competitors. The results showed that, as expected from other systems (Pieler *et al.*, 1987; Sakonju *et al.*, 1981), the 5S C box alone could compete with 5S gene transcription, presumably by binding a 5S-specific, TFIIIA-like factor, but could not compete with tRNA transcription. The presence of the tRNA A box sequence in the 5S gene did not relieve this competition. The B box of the tRNA gene is also gene-specific, and in other systems binds TFIIIC. In this study, it competed effectively with transcription of the $tRNA^{Leu}$ gene and of the 5S-tRNA hybrids containing

the $tRNA^{Leu}$ B box. It also competed effectively with 5S gene transcription, indicating that at least one factor is shared between *N. crassa* 5S and tRNA transcription. This factor is probably TFIIIC, by comparison to other systems, but the present data do not exclude other possibilities. TFIIIB also is expected to be required in common, by analogy with other systems. B box competition experiments are unlikely to detect TFIIIB since tRNA upstream sequences are required for TFIIIB binding in yeast (Stillman and Geiduschek, 1984), and the $tRNA^{Leu}$ gene with only a B box is not transcribed in *N. crassa* (data not shown).

Factors involved in selecting the transcription initiation site also appear to be common to the *N. crassa* 5S and tRNA genes. Transcription of the *N. crassa* 5S gene is initiated 50 bp upstream from the A box (measured from the first T of the consensus in Fig 1A), while transcription of the $tRNA^{Leu}$ gene is initiated about 18 bp upstream from the A box (Huiet *et al.*, 1984; Tyler and Giles, 1984). In *N. crassa*, the 5S initiation site is primarily determined by a TATA box at -29 (Tyler, 1987), in contrast to *Xenopus* where the transcription startpoint is measured from the A box (Ciliberto *et al.*, 1983; Sakonju *et al.*, 1980). The sequences determining the transcription startpoint of the *N. crassa* $tRNA^{Leu}$ gene have not been defined, but there is a TATA sequence about 28 bp upstream from the initiation site. In yeast there is evidence that the transcription factor TFIIIB binds to a TATA-like sequence around -30 of a tRNA gene, from

whence it directs transcription initiation (Kassavetis *et al.*, 1990). In the *N. crassa* 5S-tRNA hybrids, 50-60% of the transcripts were initiated from the normal 5S initiation site, even in the presence of the tRNA A box sequence and in the absence of the Ribo box, despite the fact that this initiation site is 32 bp further from the A box than in a normal tRNA gene. Thus much of the transcription initiation of the 5S-tRNA hybrid appears to be determined by the 5S TATA box and by the factors that bind to it, even though the internal elements function like those of a tRNA gene. The remaining transcripts were initiated from several sites further downstream, closer to the A box. These sites may reflect a tRNA-like preference for initiation sites closer to the A box. It was showed previously that when the 5S TATA box was moved further upstream, or was deleted, some transcription was initiated at heterogenous sites closer to the normal initiation site (Tyler, 1987). Those novel sites were located about 30 bp downstream of fortuitous TATA-like sequences. In this study, the minor transcripts from the 5S-tRNA hybrid are likewise initiated about 30 bp downstream of partial TATA sequences. Therefore, TATA sequences may normally determine the transcription initiation sites of *N. crassa* tRNA genes, as well as the 5S genes.

The A boxes of 5S and tRNA genes have generally been assumed to be functionally interchangeable (Ciliberto *et al.*, 1983; Pieler *et al.*, 1987). For example, in *Xenopus,* the somatic 5S A box matches the tRNA consensus

perfectly (Pieler *et al.*, 1987). Furthermore, Ciliberto *et al.* (1983) showed that the A boxes of a *Xenopus* 5S gene and of a $tRNA^{Pro}$ gene from *C. elegans* appeared to be interchangeable when 5S-tRNA and tRNA-5S hybrids were transcribed in *Xenopus* oocytes. However, the transcription efficiencies of the different constructs could not be directly compared in those experiments, because the structures of the transcripts were very different. In *Xenopus* and *S. cerevisiae* tRNA genes, TFIIIC binds well to the A box in a DNase I footprinting assay following binding to the B box (Fradkin *et al.*, 1989; Kassavetis *et al.*, 1990). In the 5S genes of *Xenopus*, a single base pair mutation in the A box can interfere with binding of TFIIIC to the gene (Pieler *et al.*, 1987), but no binding of TFIIIC to the A box can be detected by DNaseI footprinting (Carey *et al.*, 1986; Fradkin *et al.*, 1989). In yeast 5S genes, a DNase I footprint over the A box is generated only when TFIIIA is bound to the adjacent C box and when both TFIIIC and TFIIIB are present, and it is not known which factor makes the additional contacts with the DNA (Kassavetis *et al.*, 1990). In *N. crassa*, the finding that the 5S and tRNA A box sequences are not functionally interchangeable strongly suggests that if TFIIIC interacts directly with the 5S A box, it is doing so differently than in a tRNA gene, or else a completely different factor normally interacts with the 5S A box. The fact that *N. crassa* 5S genes require an additional element, the Ribo box, but an *N. crassa* tRNA gene does not, tends to support the possibility of a different factor. Since the Ribo box is adjacent to the A box, and must be

spaced a precise distance from it (Tyler, 1987), it is possible that a factor could bind the A box and Ribo box jointly. Since the requirement for the Ribo box and for the 5S version of the A box occurs only in the presence of the 5S C box, and is lost when the C box is replaced by the tRNA B box, it appears that transcription factor TFIIIA determines the nature of the interactions at the Ribo box and A box. This is also consistent with the observation that in the absence of the C box, neither the Ribo box nor the A box, nor the two together, can compete with transcription of a wild type 5S gene (data not shown). The present data do not distinguish between the possibilities that the factor(s) binding the 5S Ribo box and A box is TFIIIA, that it is TFIIIC (possibly with a 5S-specific subunit), or that it is a new 5S-specific transcription factor with no identified counterpart in yeast or vertebrate systems.

CHAPTER 4

FUNCTIONAL ANALYSIS OF THE RIBO BOX IN A HETEROLOGOUS GENE CONTEXT: Studies on Its Influence on *qa-2* Transcription *in vivo*

4.1. INTRODUCTION

The specificity of eukaryotic gene transcription is defined by the interaction between *cis* elements (promoter sequences) and *trans* factors (transcription factors). The three eukaryotic RNA polymerases generally utilize distinct DNA elements and transcription factors, with a couple of possible exceptions (Lorch *et al.*, 1990; Margottin *et al.*, 1991; Murphy *et al.*, 1989). Ribosomes contain components requiring transcription by all three RNA polymerases. These components are the 17S, 5.8S, and 25S rRNAs, the precursor of which is synthesized by RNA polymerase I (reviewed by Sollner-Webb and Tower, 1986); 70-80 r-proteins whose mRNAs are synthesized by RNA polymerase II (reviewed by Mermelstein *et al.*, 1989; Mitchell and Tjian, 1989; Saltzman and Weinmann, 1989; Sawadogo and Sentenac, 1990; Struhl, 1989); and 5S rRNA which is made by RNA polymerase III (reviewed by Geiduschek and Tocchini-Valentini, 1988; Palmer and William, 1990). Therefore, ribosome biosynthesis requires an

interplay among the three RNA transcription systems.

Gene specific regulation of transcription in eukaryotes is mediated by promoter-specific and general transcription factors. Upstream or downstream sequence elements are often the major determinants of the regulatory properties of a particular gene. Genes subject to a common control mechanism often contain one or more conserved upstream or downstream sequence elements through which common DNA binding regulatory proteins co-regulate their expression. Example of such genes include those encoding enzymes of amino acid biosynthesis in yeast and *Neurospora crassa* (Arndt and Fink, 1986; Hope and Struhl, 1985; Paluh *et al.*, 1988), and genes encoding ribosomal proteins (r-proteins) in yeast (Planta and Raué, 1988; Warner, 1989). Studies of over 20 r-protein genes in yeast have identified a 15 bp conserved upstream activation sequence in *Saccharomyces cerevisiae* known as UAS_{rpg}, which coordinates the transcription of most r-protein genes in response to carbon nutrition. The UAS_{rpg} is often present in duplicated copies which act synergistically to produce maximal activation (Woudt *et al.*, 1987). Deletion of the UAS_{rpg} from genes renders them inactive and unresponsive to nutritional shifts (Donovan and Pearson, 1986; Herruer *et al.*, 1987; Planta and Raué, 1988; Rotenberg and Woolford, 1986; Woudt *et al.*, 1987; Woudt *et al.*, 1986). Insertion of a synthetic UAS_{rpg} into the promoter of a non-r-protein gene results in activation of the gene with r-protein genes (Herruer *et al.*, 1987).

The UAS_{rpg} is functional in both orientations, though with unequal efficiencies. A certain minimal distance between the UAS_{rpg} and the transcription initiation site is required (Woudt *et al.*, 1986). A transcriptional activator protein variously known as TUF, RAP, or GRF binds the UAS_{rpg} and has been isolated and characterized (Buchman *et al.*, 1988a; Buchman *et al.*, 1988b; Huet and Sentenac, 1987; Shore and Nasmyth, 1987; Shore *et al.*, 1987).

In *N. crassa*, a *cis* element called the Ribo box has been identified as essential for transcription of the 5S and the 40S rRNA genes *in vitro* (Tyler, 1987; 1990). In addition, three copies of the Ribo box also occur in the promoter region of the ribosomal protein gene, *crp-2*, one of them at the transcription initiation site (Tyler and Harrison, 1990). Therefore, the Ribo box may coordinate transcription of ribosomal genes in *N. crassa* (Tyler and Harrison, 1990) by serving as a binding site for a common activator similar to the yeast UAS_{rpg} (Warner, 1989), or as a binding site for a common repressor protein (Levine and Manley, 1989). However the function of the Ribo boxes in transcription of the *crp-2* gene has not been determined *in vivo*. One way to examine the function of the Ribo box in RNA polymerase II transcription is to place the Ribo box in a non-ribosomal gene context to monitor its effect on coordinating expression of this gene with the ribosomal genes.

Though the first fungal *in vitro* transcription system for genes transcribed by RNA polymerase II was developed in *N. crassa* (Tyler and Giles, 1985), this system is relatively inefficient and r-protein genes are not transcribed by it. In the yeast *S. cerevisiae* the function of a specific gene can be routinely tested *in vivo* using gene replacement mediated by homologous recombination (Rine and Carlson, 1985). Gene replacement methods have also been developed in *Aspergillus nidulans* (Miller *et al.*, 1985a), but were not available in *N. crassa* at the time of the studies reported in this chapter.

This chapter reports the functional assay of the Ribo box *in vivo* in a heterologous gene context, the *qa-2* gene, using gene replacement techniques. The *qa-2* gene belongs to the well characterized *qa* gene cluster (Geever *et al.*, 1989; Giles *et al.*, 1985), and has been used routinely as a transformation vector in *N. crassa* (Austin and Tyler, 1990; Case *et al.*, 1979). The *qa* gene cluster comprises five structural and two regulatory genes that mediate the initial steps in quinic acid breakdown. The *qa* genes are transcriptionally induced 50- to 1,000- fold by quinic acid and are coordinately regulated by the positive and negative regulators, *qa-1F* and *qa-1S* respectively (Geever *et al.*, 1989; Giles *et al.*, 1985). Transcription of the *qa-2* gene is activated by the *qa-1F* protein which selectively binds to sites at -502, -483, -384, and -120 in the 5' flanking sequences *in vitro* (Baum *et al.*, 1987; Geever *et al.*, 1989). Using a gene replacement strategy

developed for this study, a single copy of the Ribo box was placed into the promoter of the genomic *qa-2* gene in both orientations. An identical length polylinker fragment was placed at the same location as a control. Transformants resulting from homologous recombination were selected and screened by the polymerase chain reaction (PCR) for insertion of the Ribo box or a polylinker into the *qa-2* promoter. The effect of the Ribo box insertion on *qa-2* gene transcription *in vivo* was then assayed.

4.2. MATERIALS AND METHODS

4.2.1. Strains and plasmids. The *N. crassa* strain used for the studies was 246-89601-2A (A *qa-2 aro-9 inl*) (Case *et al.*, 1977). All plasmids used for the gene replacement studies derived from a 951 bp *BglII-SphI* fragment from the *qa-2* gene (-468 to +484) subcloned into pUC13-1 (pQa2Bg/Sp). An 18 bp double stranded oligonucleotide matching the Ribo boxes of the 5S and *crp-2* genes (Tyler, 1987; Tyler and Harrison, 1990) (Fig 1A) was inserted into a unique *BstBI* site at -183 in one (pYQaD1L and pYQaD1R), two (pYQaD2LHT and pYQa2RTT), or multiple copies (pYQaDM) (Fig 1B). An 18 bp polylinker sequence from pUC13-1 was inserted at the same site as a control. The polylinker sequence used for the insertion is shown in Fig 1A. The insertion was carried out as follows. A 281 *BstBI-BglII* fragment end-filled at the *BstBI* site from plasmid pQa2Bg/Sp was ligated into pUC13-1 which was digested with *SmaI* and

BglII, resulting in a new plasmid named "intermediate". Insertion of the 18 bp pUC13 polylinker was then achieved by ligating a 299 bp *BglII-AccI* fragment from the intermediate plasmid back to pQa2Bg/Sp which has been digested with the *BglII* and *BstBI*. The resulting plasmid was named pYQapoly (Fig 1B). The insertions in all plasmids were confirmed by sequencing the region by chemical cleavage (Maxam and Gilbert, 1980) with a modification (Shi and Tyler, 1989).

4.2.2. Transformation of *N. crassa*. Transformation was carried out using methods essentially as described (Austin and Tyler, 1990). All plasmids (shown in Fig 1) used for transformation were linearized with *BglII* at -468 of the *qa-2* gene. Briefly, 5 μg of plasmid DNA was incubated with 10^7 spheroplasts on ice for 20 min in 110 μl of 1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris-HCl (pH7.4), 8% polyethylene glycol (MW3350), 1% dimethyl sulfoxide, 50 μg/ml heparin; followed by addition of 1 ml of 40% polyethylene glycol (MW3350), 50 mM $CaCl_2$, 50 mM Tris-HCl (pH7.4) and incubated at room temperature for 20 min or longer. Plating was carried out by mixing the spheroplast mixture with 15 ml SFG medium (preincubated at 50 °C, containing Vogel's minimal medium with 2% sorbose, 0.05% glucose, 0.05% fructose, 50 μg/ml inositol, and 1.5% agar) and immediately plated onto 15 cm petri dishes containing preset SFG medium containing 2.5% agar and 1 M sorbitol.

4.2.3. Mini-prep of *N. crassa* genomic DNA and identification of transformants with PCR. A simple and quick method was developed to extract DNA from *N. crassa* conidia grown in a test tube. The mini-prep was carried out as follows. Conidia from each test tube were harvested individually into 2 ml sterile distilled water, poured into a 1.7 ml eppendorf tube, and collected by centrifugation for 5 min in a bench top microcentrifuge at 10,000 rev./min. The conidial pellet was resuspended in 300 µl extraction buffer (2% SDS, 25 mM EDTA, 250 mM NaCl, 200 mM Tris-HCl (pH 8.5)), and incubated at 100 °C for 2 min. 300 µl of phenol saturated with the extraction buffer was added to the heated conidia, and vortexed for 1 min, followed by a 1 min centrifugation using the microfuge at top speed. The aqueous phase was transferred to a new tube. The conidia preparation was extracted once more by adding 200 µl of extraction buffer, mixed by vortex action, centrifuged for 1 min, and the aqueous phase combined with that from the first extraction. The combined aqueous phases were then extracted 2-3 times with 500 µl of phenol saturated with extraction buffer, and extracted once with 500 µl of chloroform, followed by addition of 0.6 vol of isopropanol to precipitate the genomic DNA. The DNA pellet was then washed with 80% ethanol, dried under vacuum, and dissolved in 50 µl TE buffer (10 mM Tris (pH 7.4), 1 mM EDTA). Typically, the mini-prep yielded about 1 µg of total genomic DNA which could be used for 5-10 reactions as a template for the polymerase chain reaction (PCR).

FIG. 1. Oligo nucleotide sequences and plasmid structures. *A*. Ribo box and polylinker sequences for insertion at the -183 site of *qa-2* gene. *B*. Insertion sequences at the –183 *BstBI* site of plasmids used for transformation. *C*. Primer sequences used for PCR amplification of the 1 kb genomic fragment spanning the Ribo box insertion site.

A

CRP-2 RIBO BOX (-9)	5'	CTGGAAAACTCGGGATC	3'	
5S RIBO BOX	5'	CTGGAAAACTGGAGATC	3'	
RIBO BOX CONSENSUS	5'	CGCTGGAAAACTCGAGAT GACCTTTTGAGCTCTAGC	3'	
POLYLINKER	5'	ACTCTAGAGGATCCCCCG TGAGATCTCCTAGGGGGC	3'	

B

INSERT STRUCTURE	**PLASMID NAME**
	pYQapoly
	pYQaD1L
	pYQaD1R
	pYQaD2LHT
	pYQaDM2RTT
	pYQaDM

C

5' PRIMER	(-490)	5' GAGGTGATGCACGGGTAGTCG 3'		(-470)
3' PRIMER	(+508)	5' ATGTCTGAACGCCTCTCGCGC 3'		(+488)

PCR amplification was used to identify the transformants which carried a Ribo box insertion or the polylinker sequence at the *BstBI* site of the *qa-2* gene. About 200 ng (10 µl) genomic DNA from a conidial mini-prep of a given transformant was used as template DNA for the amplification. The primer sequences (Fig 1c) used for the amplification derived from sequences at -468 just upstream of the *BglII* site and +484 just downstream of the *SphI* site of the *qa-2* gene. Therefore, PCR reactions using the primers would not amplify *qa*-2 sequences located within the transformation vector. Conditions used for the DNA amplification reactions were basically as described (Saiki *et al*., 1988). The PCR reaction was carried out in a programmable DNA thermal cycler (Perkin Elmer Cetus). The thermal program used for PCR was 94 °C for 2 min, followed by 25 cycles of "1 min at 94 °C, 2 min at 50 °C, and 3 min at 72 °C", and finally 72 °C for 10 min. The PCR amplification normally yielded more than 2 µg DNA fragment, one tenth of which was electrophoresed in a 1.5% agrose gel to identify the presence of an insertion sequence. Amplified DNA from preselected transformants was then digested with *XhoI*, *XbaI*, and *BstBI* respectively to confirm the insertion of a Ribo box (which added a new *XhoI* site), the polylinker (which added a new *XbaI* site), or no insertion (which retained a *BstBI* site). The same procedures were also used to identify homokaryons of selected transformants.

4.2.4. Isolation and quantitation of mRNA. To isolate mRNA from

transformants grown under different nutritional conditions, conidia (800 ml of 10^6/ml) from each transformant were shaken at 300 rev./min at 30 °C overnight without light in minimal Vogel's medium (Vogel, 1964) plus 1.5% sucrose to allow germination and outgrowth. After 13.5 hours, each culture was harvested by filtration onto Miracloth (Calbiochem), rinsed with cold sterile distilled water, and then divided into three equal portions. Portion 1 (S3) was reinoculated into 1.5% sucrose medium for 3 hours. Portion 2 (Q3) and portion 3 (Q10) were reinoculated into minimal Vogel's medium plus 0.3% quinic acid (Giles *et al.*, 1985) for 3 and 10 hours respectively. Finally, the mycelia were harvested by filtration onto miracloth, rinsed with cold sterile distilled water and frozen immediately in liquid N_2. mRNA was prepared from the frozen mycelia using the methods described by Patel *et al.* (1981). Briefly, 5 g of crushed frozen mycelia were homogenized for 1 min with a probe homogenizer (Virtis) in 100 ml each of extraction buffer (0.1 M sodium acetate (pH 5.0), 10 mM EDTA, 4% SDS) and PCA (49% phenol, 0.1% 8-hydroxyquinoline (w/v), 49% chloroform, 2% isoamyl alcohol (v/v)). The aqueous phase was extracted twice with PCA and chloroform, and digested with proteinase K (1 mg/ml) for 1 hour at room temperate with gentle shaking (<100 rpm). The digested solution was extracted once more with chloroform and the total RNA was precipitated with 3 vol of ethanol. The RNA pellet was dissolved in 5 ml of TES buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.2 Sarkosyl). The total RNA was adjusted to 0.5 M NaCl and then heated at 65 °C for 5 min

before passing through an oligo (dT)-cellulose column. The column was then washed twice with NTES buffer (0.5 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 0.2% Sarkosyl). The mRNA was eluted from the column by TES buffer, and was concentrated by precipitation with 3 vol of ethanol.

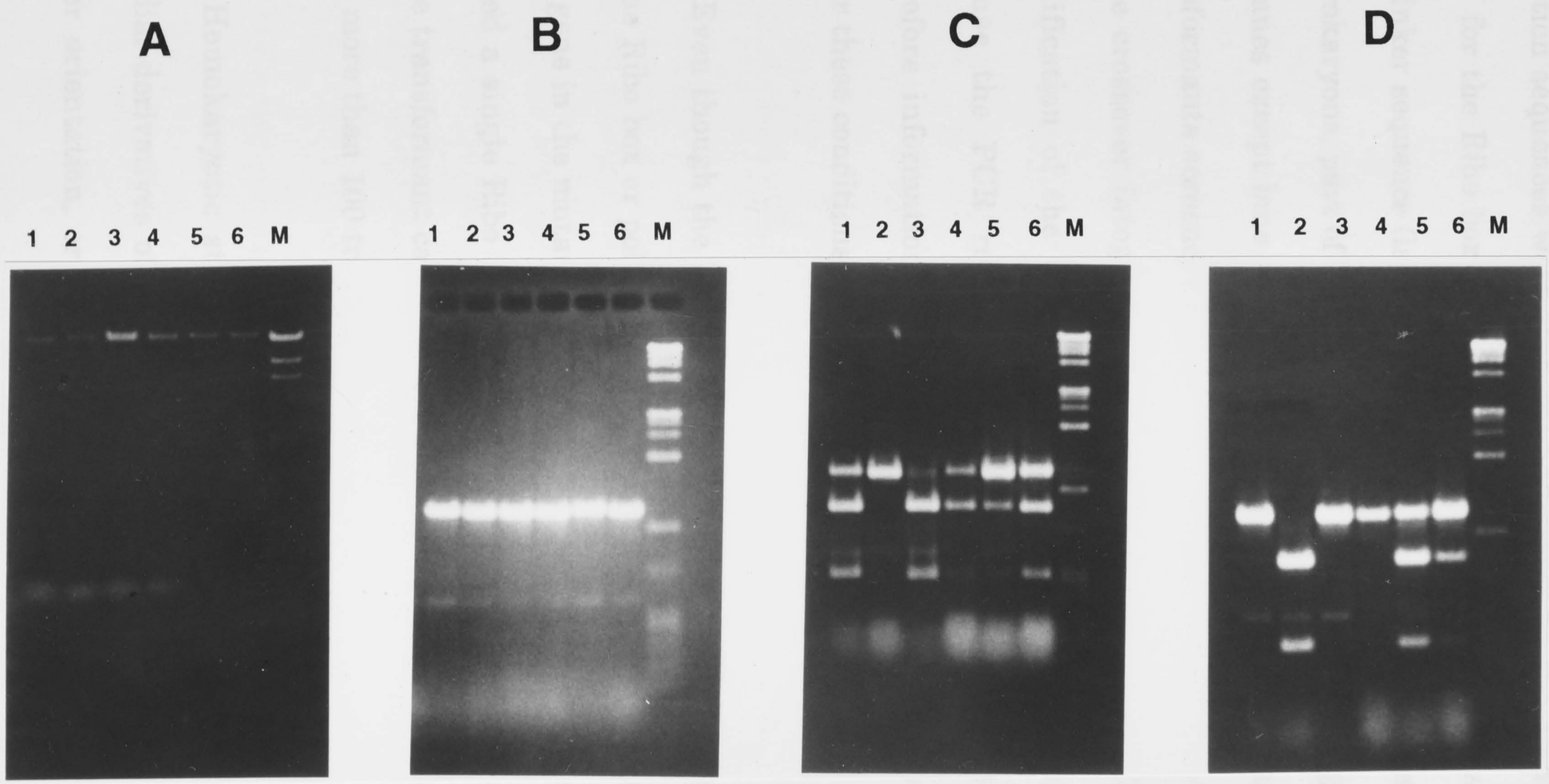
S1 nuclease mapping was used to measure the mRNA levels of the *qa-2* ,*qa-4*, *qa-y*, *crp-3*, and β–tubulin genes. S1 mapping was carried out as described (Tyler and Harrison, 1990) except that hybridization was at 58 °C. The fragments used for the S1 analysis were a 430 bp *AvaI-SalI* fragment (3' end labelled) for *qa-2* (Geever *et al.*, 1989), a 430 bp *HindIII* fragment (3' end labelled) for *qa-4* (Geever *et al.*, 1989), a 337 bp *PvuII-HindIII* fragment (5' end labelled) for *qa-y* (Geever *et al.*, 1989), a 373 bp *BamHI-AvaI* fragment (5' end labelled) for *crp-3* (Chapter 5), and a 353 bp *PvuII-BamHI* fragment (3' end labelled) for the β–tubulin gene (Orbach *et al.*, 1986). The mRNA level for each gene was measured by scanning the corresponding radioactive band in the dried gels using an AMBIS radioanalytic imaging system (San Diego, CA).

4.3. RESULTS

The Ribo box was placed in the promoter region of the *N. crassa qa-2* gene by a novel gene replacement strategy using a well developed

transformation system and the *qa-2* gene as a vector. The choice of *qa-2* provides several advantages for the functional assays of the Ribo box *in vivo*: the *qa-2* gene, its genetics and its regulation are well characterized (Baum *et al.*, 1987; Geever *et al.*, 1989; Giles *et al.*, 1985); the gene is used routinely for transformation (Austin and Tyler, 1990; Case *et al.*, 1979); and expression of the gene is inducible by quinic acid (Giles *et al.*, 1985). To insert the Ribo box into the genomic *qa-2* gene, plasmids that carried a 3'-truncated wild type fragment of the *qa-2* gene (from -468 to +484) with a Ribo box inserted at -183 were used as vectors to transform strain 246-89601-2A which carries a frame-shift mutation at codon 8 of the *qa-2* gene. The Ribo box was inserted in single or multiple copies into the *BstBI* site of the *qa-2* gene on the vector (Fig 1). An identical length polylinker sequence from plasmid pUC13-1 was inserted at the same site to form a control plasmid (Fig 1). Only transformants that result from gene replacement spanning the frame-shift mutation could grow on minimum medium without aromatic amino acids (*qa-2* can complement an *arom-9* mutation) (Case *et al.*, 1979). PCR amplification was used to screen mini-prep genomic DNA (Fig 2A) for insertion of the Ribo box or polylinker sequences, using primers (Fig 1C) spanning the insertion site. Transformants carrying the insertion sequences were identified from the sizes. The amplified fragment was 1 kb from transformants without an insertion at the *BstBI* site (all lanes except lane 5 in Fig 2B), or 18 bp longer

FIG. 2. PCR amplification of genomic *qa-2* fragment. M: DNA size marker; a mixture of *HindIII* digested λ phage DNA and *HaeIII* digested of M13 phage DNA. *A*. Examples of mini-prep genomic DNA from conidia of different transformants. *B*. PCR amplified genomic DNA fragments (1 kb) from six transformants (different from A) resolved on 1.5% agrose gel. Lane 5 indicates insertion of a single Ribo box sequence. *C*. *XbaI* (lane 1) and *XhoI* (lanes 5-6) digests of PCR amplified genomic DNA fragments from six transformants (different from A and B, same as D). The top band (except lane 2) is not due to partial digestion but instead reflects wild-type *qa-2* DNA in heterokaryons. Lanes differ in the presence of insertion sequences at the *BstBI* site in the *qa-2* gene; 1: polylinker sequence; 2: no insertion; 3: a single Ribo box (3' to 5'); 4-6 (different isolates): a single Ribo box (5' to 3'). *D*. *BstBI* digestion of the same DNAs as in *C*.


A
1 2 3 4 5 6 M
B
1 2 3 4 5 6 M
C
1 2 3 4 5 6 M
D
1 2 3 4 5 6 M

with a single insertion sequence (lane 5, Fig 2B). The identities of the insertion sequences were confirmed by digesting the amplified DNAs with *XhoI* for the Ribo box sequence (lanes 3-6, Fig 2C) or with *XbaI* for the polylinker sequence (lane 1, Fig 2C). Since most of the transformants were heterokaryons, part of the amplified DNA still could be digested with *BstBI* (all lanes except lane 2, Fig 2D). It should be noticed that among all the transformants screened by PCR, not a single transformant resulting from single crossover integration was detected. This could be due to biased amplification of the wild-type sequences in DNA from heterokaryons, because the PCR reaction favors amplification of short sequences. Therefore information on single crossover may simply have been lost under these conditions.

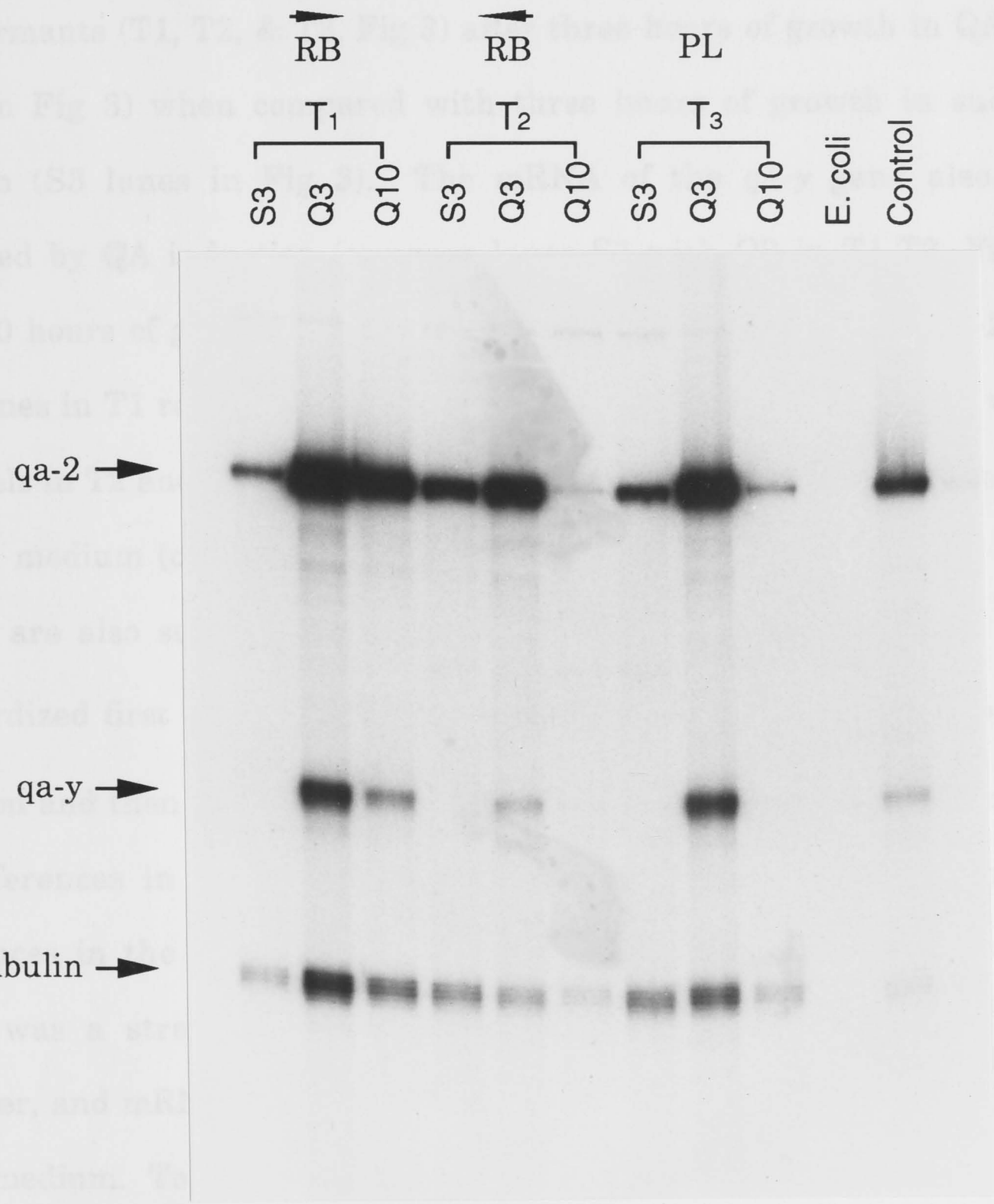
Even though the distance was only 293 bp between the insertion site for the Ribo box or polylinker sequences and the point mutation site of the *qa-2* gene in the mutant strain, less than 5% of the transformants screened carried a single Ribo box or the polylinker sequences. In addition, not a single transformant carrying multiple copies of the Ribo box was identified from more than 100 transformants screened by PCR.

Homokaryotic strains were selected by PCR screening of single conidial derivatives of transformants which carried a single Ribo box in either orientation, or a polylinker sequence. These strains were then

grown under different nutritional conditions to study the regulatory effect of the Ribo box on *qa-2* transcription. Conidia from each transformant were grown for 13.5 hour in 1.5% sucrose medium then divided into three portions. Portion 1 was reinoculated into 1.5% sucrose medium and cultured for 3 hours (S3). Portions 2 and 3 were inoculated into 0.3% quinic acid (QA) medium and cultured for 3 (Q3) and 10 hours (Q10) respectively. The regulatory effect of the Ribo box was examined by comparing the relative mRNA levels of the *qa-2* gene carrying a Ribo box with that of the *qa-2* gene carrying a polylinker sequence under each growth condition. Three categories of genes were used as controls for the relative mRNA level of the *qa-2* gene. These were the *qa-4* and the *qa-y* from the *qa* gene cluster (Geever *et al.*, 1989) whose expression is inducible by quinic acid (Baum *et al.*, 1987), a r-protein gene, *crp-3* (Chapter 5), and a "house keeping" gene, β–tubulin (Orbach *et al.*, 1986), whose expression is relatively constant under different growth conditions. *Qa-y* is transcribed convergently with *qa-2* and its promoter is approximately 7.8 kb upstream of the site of the Ribo box insertions. Therefore its transcription should be relatively unaffected by the insertions. Qa-4 also is transcribed convergently with *qa-2* and its promoter is about 2.7 kb upstream of the Ribo box insertion site.

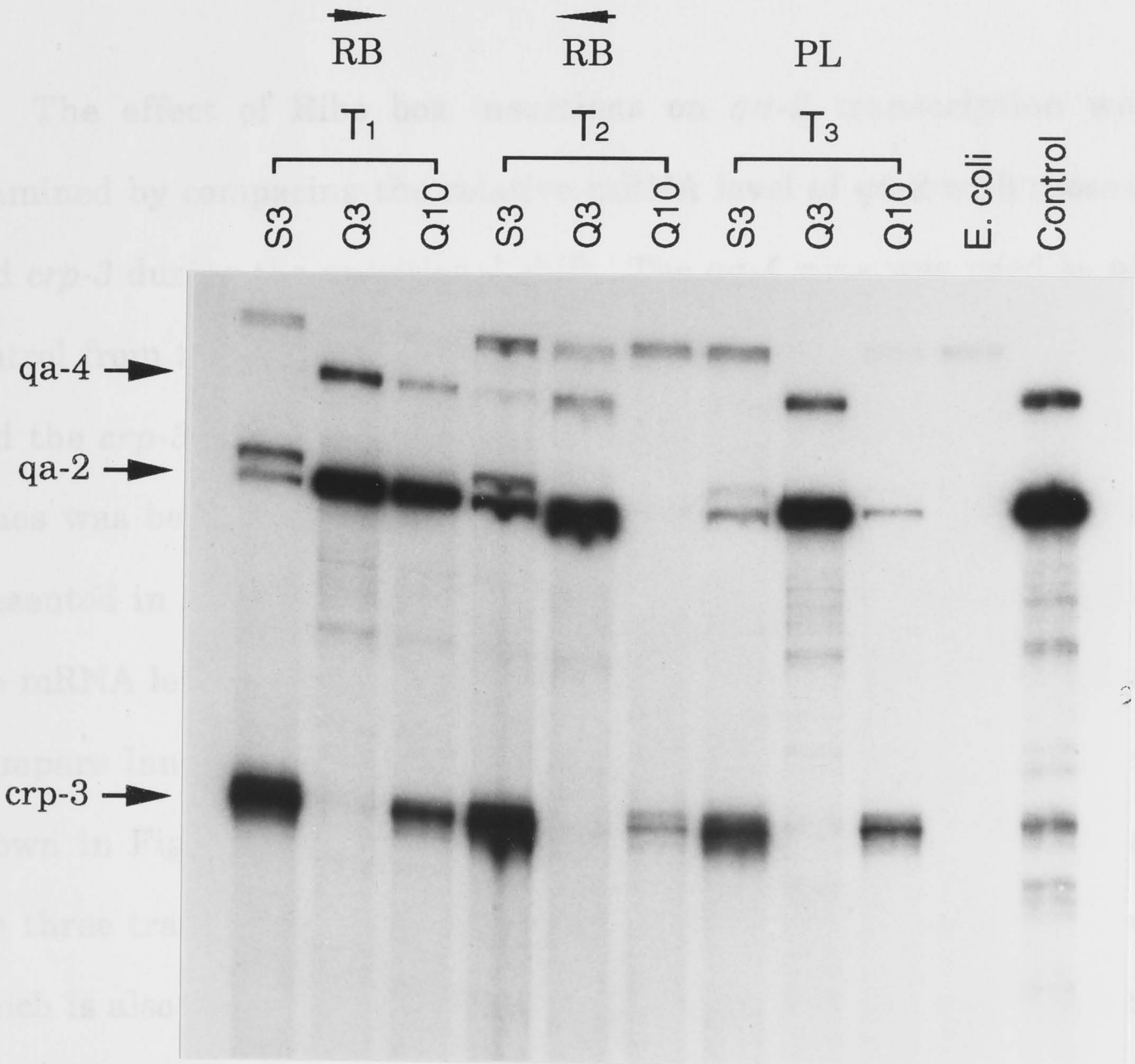
Fig 3 shows the change in mRNA levels of *qa-2* gene in comparison to

FIG. 3. Effect of Ribo box insertion on *qa-2* expression under QA induction: comparison to *qa-y* and β–tubulin genes. Quantitation of mRNAs by S1 nuclease digestion of DNA.RNA hybrids was carried out as described in Material and Methods. The mRNA was isolated from three *N. crassa* transformants (T1, T2, &T3) grown in sucrose for three hours (S3), or in quinic acid for 3 hours (Q3) and 10 hours (Q10) respectively. Arrows at the top indicate orientation of the Ribo box (RB) insertion. PL: polylinker insertion. E. coli: control RNA from *E. coli*. Control: control RNA from 6 hour QA induction of a *N. crassa* strain which did not carry an insertion sequence at the -183 site of the *qa-2* gene. The DNA probes used were fragments from the *qa-2*, *qa-y*, and β–tubulin genes (see Material and Methods). The respective fragments protected from S1 nuclease are marked by the arrows at the left side.


RB
RB
PL
T1
T2
T3
S3
Q3
Q10
S3
Q3
Q10
S3
Q3
Q10
E. coli
Control
qa-2
qa-y
β-tubulin

those of *qa-y* and β–tubulin genes during the shift from sucrose to QA among three different transformants. The relative mRNA level of the *qa-2*, standardized to that of β–tubulin, was increased 5 fold in all three transformants (T1, T2, & T3, Fig 3) after three hours of growth in QA (Q3 lanes in Fig 3) when compared with three hours of growth in sucrose medium (S3 lanes in Fig 3). The mRNA of the *qa-y* gene also was increased by QA induction (compare lanes S3 with Q3 in T1-T3, Fig 3). After 10 hours of growth in QA, the relative mRNA levels of the *qa-2* and *qa-y* genes in T1 remained high (compare Q10 with Q3 in T1, Fig 3), while the levels in T2 and T3 had unexpectedly decreased to levels below those in sucrose medium (compare lanes Q10 with S3 in T2 and T3, Fig 3). The results are also summarized in Table 1. All the data in the table were standardized first with that of β–tubulin gene to account for lane-to-lane variation and then with the control strain (lane "control", Fig 3) to account for differences in the specific activities of the probes and the normal differences in the absolute levels of the three *qa* mRNAs. The control strain was a strain which did not carry any insertions in the *qa-2* promoter, and mRNA was prepared from the strain after 6 hour of growth in QA medium. To elucidate any regulatory effect of the Ribo box insertion on *qa-2* transcription, the mRNA level of *qa-2* was compared to that of *qa-y* among the three transformants. As shown in Table 1 that the ratio of *qa-2* to *qa-y* was significantly higher in Q3 of T2 when compared with that in T1

FIG. 4. Effect of Ribo box insertion on *qa-2* expression after QA induction: comparison to *qa-4* and *crp-3* genes. Symbols and markings are the same as in Fig 3 except that the DNA probes used for the S1 mapping were fragments from *qa-2*, *qa-4*, and β–τubulin genes (see Material and Methods).


RB
RB
PL
T1
T2
T3
S3
Q3
Q10
S3
Q3
Q10
S3
Q3
Q10
E. coli
Control
qa-4
qa-2
crp-3

or T3. However, this effect was not observed from 3 hours of growth in sucrose (S3 of T2) or 10 hours of growth in QA (Q10 of T2) of the transformant. moreover the effect appears to be due to the exceptionally low mRNA level of *qa-y* gene (qa-y/β-tub,Table 1) measured in T2, rather than any activation effect caused by the Ribo box insertion (see below).

The effect of Ribo box insertions on *qa-2* transcription was also examined by comparing the relative mRNA level of *qa-2* with those of *qa-4* and *crp-3* during the nutritional shift. The *qa-4* gene was used as another control from the *qa* gene cluster to verified the results presented in Fig 3, and the *crp-3* gene was used to confirm that expression of the r-protein genes was being regulated under the growth conditions. The results are presented in Fig 4 and are also summarized in Table 1. Fig 4 shows that the mRNA levels of *qa-2* and *qa-4* are coordinately induced by quinic acid (compare lanes S3 with Q3, T1–T3), which is consistent with the results shown in Fig 3. Similarly, no significant differences were found among the three transformants in mRNA levels of *qa-2* relative to those of *qa-4*, which is also indicated in Table 1 as the ratios of *qa-2* to *qa-4*. The ratio of *qa-4* to β–tubulin in Table 1 was calculated from the ratio of *qa-2* to β-tubulin in Fig 3 and the ratio of *qa-2* to *qa-4* in Fig 4. Fig 4 also demonstrated that expression of the r-protein gene, *crp-3*, was strongly regulated under the nutritional shift. In contrast to the *qa* genes, the

TABLE 1. Quantitation of relative mRNA levels of *qa* genes. All data originated from quantitation of the radioactive bands in Fig 3 and 4. The data were standardized to β-tubulin (as the ratio of each gene to β–tubulin) for each strain and growth conditions. The ratios of *qa-4* to β–tubulin were calculated from the ratio of *qa-2* to β–tubulin in Fig 3 and the ratio of *qa-2* to *qa-4* in Fig 4. The relative mRNA levels of the *qa* in the control strain were standardized to 1.0 to aid comparisons. The blanks in the table are due to very weak radioactive bands in the Figs 3 and 4. All symbols are same as in Fig 3 and 4.

TABLE 1

STRAINS / RATIOS	CONTROL	T1			T2			T3		
		S3	Q3	Q10	S3	Q3	Q10	S3	Q3	Q10
qa-2/β-tubulin	1.00	0.13	0.60	0.47	0.30	1.40	0.08	0.13	0.67	0.06
qa-4/β-tubulin	1.00		0.67	0.28	0.14	0.94			0.59	
qa-y/β-tubulin	1.00		0.59	0.30		0.29			0.88	
qa-2/qa-4	1.00		0.89	1.65	2.14	1.49			1.15	
qa-2/qa-y	1.00		1.01	1.53		4.90			0.76	

mRNA level of *crp-3* decreased dramatically after three hours of growth in QA (compare lanes S3 with Q3, T1–T3). The *crp-3* mRNA level had partially recovered after 10 hours growth in QA (compare lanes Q10 with Q3, T1–T3). This suggests that the insignificant effect of the Ribo box insertion on *qa-2* expression was unlikely to be due to a lack of response by ribosomal genes under the growth conditions.

4.4. DISCUSSION

The *qa-2* gene has been used routinely as a selectable marker for transformation (Austin and Tyler, 1990; Case *et al.*, 1979). In this study, it also is used as a reporter gene for the functional assay of the Ribo box. The Ribo box was inserted in single or multiple copies at the *BstBI* site at -183 in a *qa-2* 3' deletion mutant on a plasmid vector. An identical length polylinker sequence was inserted at the same site in a control plasmid. In contrast to the high rate of homologous integration over random integration in yeast, the majority *N. crassa* transformants result from heterologous integration. The actual frequency of homologous integration depends on the length of the homologous fragment on a vector (Asch and Kinsey, 1990), ranging from 3% to 15% of the total transformants. Here, a selective medium (minimal medium) was used to obtain transformants which resulted from homologous integration or gene conversion, thus repairing the endogenous *qa-2* gene. Less than 5% of the *qa-2+* colonies had

co-integrated a single copy of the Ribo box or the polylinker insertion, even though the distance between the insertion site and the mutation site was only 293 bp compared to 670 bp distal to the insertion site. Furthermore, not a single transformant which carried more than one copy of the Ribo box was identified from intensive screening of transformants from many rounds of transformation. This suggests that insertion of more than 18 bp at the -183 site could have an negative effect on the transcription of the *qa-2* gene. In fact, the insertion site is located between the two adjacent binding sites for *qa-1F* protein at -120 and- 384. This may account partly for the lack of transformants which carried more than one Ribo box sequences. However, this may not be the major cause, as a *qa-2* gene which carried a rearranged promoter upstream of -190 was still responsive to QA induction (Geever *et al.*, 1989; Geever *et al.*, 1986). Moreover, evidence from mutational studies on the *qa-2* gene *in vivo* have suggested that multiple binding sites of the *qa-1F* protein in the region upstream of -190 of the *qa-2* promoter was associated with repression of basal level transcription rather than induction (Baum *et al.*, 1987; Geever *et al.*, 1986).

The Ribo box insertion in either orientation did not significantly alter the mRNA levels of the *qa-2* gene during growth on sucrose or under QA induction when compared with polylinker insertion. Even though the ratio of *qa-2* to *qa-y* was significantly higher in transformant T1 (which carried a 3'to 5' insertion of a single Ribo box) after three hours culture in QA, this

was primarily because the *qa-y* mRNA level was aberrantly low. Moreover the effect was not observed in comparison to the *qa-4* mRNA level. Therefore, it is not likely that the Ribo box insertion had an activation effect on the *qa-2* transcription. The higher ratio may simply reflect experimental variation, since the stoichiometry among the *qa* genes is not exact at any given growth stage (Geever *et al.*, 1983) and the experiment was not replicated.

Fortuitously, the polylinker sequence from pUC13 matches part of the Ribo box sequence. However it is unlikely that this accounts for the apparently insignificant effect of the Ribo box insertion on *qa-2* transcription, since the ratio of *qa-2* to *qa-4* mRNAs in all three transformants was similar to that in the control strain after 6 hours of QA induction (Fig 4 and Table 1). Only a five fold induction of *qa-2* transcription was observed comparing QA-grown and sucrose-grown cultures of the transformants. Since the transformants retain an *arom-9* mutation, which leads to an internal accumulation of the inducer dehydroquinic acid, substantial constitutive expression of *qa-2* is expected in sucrose cultures (Chaleff, 1974; Giles *et al.*, 1985).

The level of *crp-3* mRNA was also examined to verify whether the lack of response to Ribo box insertion in the *qa-2* gene was due to non-regulated expression of r-protein genes under the growth conditions. The results

demonstrated that the expression of the *crp-3* gene was strongly regulated under these conditions. If Ribo box alone was responsible for the regulation, its influence should have been reflected in the level of *qa-2* expression.

The Ribo box is essential for 5S and 40S transcription *in vitro* (Tyler, 1987; 1990). It also is present three times in the r-protein gene *crp-2* (Tyler and Harrison, 1990), suggesting a possible role for the Ribo box in coordinating transcription of ribosomal genes. The evidence from this study indicates that the Ribo box alone cannot influence a heterologous gene promoter either positively or negatively, under the conditions tested. This finding coincides with the observation that the Ribo box is not required for transcription *in vitro* when placed in the promoter region of a $tRNA^{Leu}$ gene (Chapter 3; Shi and Tyler, 1991). It should be noted though that the failure to introduce more than one copy of the Ribo box into the *qa-2* promoter may indicate a negative effect by the Ribo box. However a control experiment using a long polylinker sequence was not carried out to show that the effect was Ribo box-specific. The Ribo box alone is not capable in interacting with transcription factors *in vitro* in a gel retardation assay (Chapter 2). In addition, transcription of the 5S gene is not sensitive to competition by the *N. crassa* 40S gene which carries an functionally exchangeable Ribo box (Tyler, 1987; B. M. Tyler, unpublished data). Therefore, all the evidence accumulated so far do not favor the model that

the Ribo box serves as an independent binding site for a transcription activator, like the *qa-1F* protein of *N. crassa* or like TUF in yeast (Baum *et al.*, 1987; Huet and Sentenac, 1987), or as an independent binding site for a repressor. It is also possible that the Ribo box may have no function in RNA polymerase II transcription at all.

CHAPTER 5

COORDINATE EXPRESSION OF RIBOSOMAL PROTEIN GENES AND IDENTIFICATION OF CONSERVED PROMOTER ELEMENTS

5.1. INTRODUCTION

Ribosomes consist of 60-80 ribosomal proteins and 3-4 rRNA molecules. Their synthesis comprises a major metabolic investment by cells. Under favorable growth conditions, ribosomes contain about 85% and 15% of the total cellular RNA and protein respectively (Alberghina and Sturani, 1981; Kief and Warner, 1981; Mager, 1988; Nomura *et al.*, 1984; Warner, 1989). Therefore regulation of the levels of ribosome synthesis and coordinate expression of individual ribosomal component genes are essential. In *E. coli* and yeast (*Saccharomyces cerevisiae*), the ribosome content is proportional to growth rate (Kief and Warner, 1981; Kjeldgaard and Gausing, 1974) and the rates of ribosomal synthesis are promptly adjusted to changes in cell growth and environmental conditions (Nomura *et al.*, 1984; Warner, 1989). In *E.coli*, the rate of ribosome synthesis is controlled by the rate of rRNA synthesis; ribosomal protein synthesis is

coordinated with rRNA synthesis by autogenous control of the translation of the ribosomal protein mRNAs (Nomura *et al.*, 1984). In eukaryotes, coordinate control of the rates of rRNA and r-protein synthesis (Kief and Warner, 1981; Mager, 1988; Warner and Gorenstein, 1978; Warner and Gorenstein, 1977) is more complex, involving genes transcribed by the three nuclear RNA polymerases. The large rRNA precursors are synthesized by RNA polymerase I, r-protein mRNAs by RNA polymerase II, and 5S rRNAs by RNA polymerase III. Coordination appears to be achieved via two basic mechanisms: firstly, there may be concerted *de novo* synthesis of the different rRNAs and r-protein mRNAs (Kief and Warner, 1981). Secondly, a variety of feedback mechanisms controlling RNA splicing and stability, and protein turnover may act to precisely balance the supply of free ribosomal components (Warner *et al.*, 1985).

In yeast, transcription of r-protein genes is coordinated during nutritional shifts of carbon or nitrogen source or amino acid starvation (Kief and Warner, 1981; Kraig *et al.*, 1982; Pearson and Haber, 1980; Warner and Gorenstein, 1978; Warner and Gorenstein, 1977). Comparison of sequenced yeast r-protein genes identified a 15 bp conserved sequence, UAS_{rpg}, in the 5' flanking region of most but not all r-protein genes (Mager, 1988; Planta and Raué, 1988; Warner, 1989). Promoter deletion and gene fusion studies of several genes confirmed that the UAS_{rpg} was responsible for coordinate activation of r-protein genes (Donovan and

Pearson, 1986; Herruer *et al.*, 1987; Rotenberg and Woolford, 1986; Teem *et al.*, 1983). The UAS_{rpg} also is found 5' to the gene encoding a common subunit of RNA polymerase I and polymerase III (Mann *et al.*, 1987), suggesting a possible mechanism for coordinating the synthesis of r-proteins with that of the rRNAs. However, this has not yet been tested, and the UAS_{rpg} is found also in many other genes not associated with protein synthesis (Warner, 1989). A regulatory protein known as TUF, RAP or GRF which binds to the UAS_{rpg} has been identified and characterized (Buchman *et al.*, 1988a; Buchman *et al.*, 1988b; Huet and Sentenac, 1987; Shore and Nasmyth, 1987; Shore *et al.*, 1987). Autogenous control of splicing of the pre-mRNA for yeast ribosomal protein *L32* has been described (Warner *et al.*, 1985; Wittekind *et al.*, 1990) but this mechanism does not appear to be general.

In mammalian systems, the promoter regions essential for efficient transcription of r-protein genes are often very short (Dudov and Perry, 1986), and share some common features. These include the lack of a canonical TATA box, a cap site embedded in a pyrimidine tract flanked by GC rich sequences, and one or two intragenic elements in the first exon or intron (Dudov and Perry, 1986; Hariharan *et al.*, 1989; Hariharan and Perry, 1989; Hariharan and Perry, 1990; Meyuhas and Klein, 1990). These common features suggest that expression of these genes may be

coordinated via one or several of the common promoter elements. But in general, co-ordinate regulation of r-protein genes in mammalian systems is not as well understood as in yeast. Translational control of r-protein synthesis has been reported in higher eukaryotes, such as *Drosophila* (Kay and Jacobs-Lorena, 1985; Schmidt *et al.*, 1985), *Xenopus* (Mariottini and Amaldi, 1990; Pierandrei-Amaldi *et al.*, 1985), and mouse (Kay and Jacobs-Lorena, 1985; Meyuhas *et al.*, 1987), but this control is not autogenous.

In *Neurospora crassa*, the rates of both rRNA and r-protein synthesis increase markedly within 30 min following a nutritional upshift from acetate to glucose. During a downshift from glucose to glycerol, both rRNA and r-protein synthesis are severely inhibited for at least two hours after the shift before a slower rate of synthesis recommences (Alberghina *et al.*, 1975; Alberghina *et al.*, 1978; Sturani *et al.*, 1976; Sturani *et al.*, 1973). Studies of 5S and 40S transcription *in vitro* have led to the identification a common *cis* element which is essential for *in vitro* transcription of the 5S and 40 rRNA genes (Tyler, 1987; 1990), but not tRNA genes (Chapter 3; Shi and Tyler, 1991). This element, the Ribo box, also is present at three locations in the promoter of a r-protein gene (*crp-2*), including the transcription initiation site (Tyler and Harrison, 1990). Therefore, the Ribo box may play a role in coordination of transcription of ribosomal genes in *N. crassa* . However, there have been no studies of how r-protein genes are regulated *in vivo*. This chapter reports the cloning and characterization of

the r-protein gene *crp-3*, homologous to yeast *rp51* and mammalian *S17*, and show that the mRNAs of four *N. crassa* r-protein genes are coordinately regulated during a nutritional downshift. Sequence comparisons of the 5' flanking regions of the *crp-1*, *crp-2* and *crp-3* genes reveal two conserved promoter elements.

5.2. MATERIALS AND METHODS

5.2.1. Amplification of a *crp-3* genomic fragment by the polymerase chain reaction (PCR). Two regions highly conserved between yeast *rp51* and human *S17* r-protein sequences were used to design degenerate oligonucleotide primers for PCR. The strongly biased codon usage of actively expressed genes in *N. crassa* was used to eliminate many possible codons. Total genomic DNA (1 μg) from *N. crassa* strain 105C was used as template DNA for PCR amplification of *crp-3* genomic fragments. Conditions used for DNA amplification reactions were essentially as described by (Saiki *et al.*, 1988). The PCR reaction was carried out in a programmable DNA thermal cycler (Perkin Elmer Cetus). The thermal program used for PCR was 94 °C for 2 min, followed by 25 cycles of 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C, and finally 72 °C for 10 min. The amplified fragments were then gel purified and used as probes for screening both cDNA and genomic libraries from *N. crassa*.

5.2.2. Cloning and sequencing of *crp-3* cDNA and genomic DNA clones. Fragments of the yeast *rp51* gene (provided by M. Rosbash, Brandeis University) and *N. crassa crp-3* genomic fragments produced by PCR were used as probes to screen cDNA and genomic libraries. The two cDNA libraries (provided by M. Sachs, Stanford University) were prepared with mRNA from either mycelia or germinating conidia, using λ-ZAP (Stratagene) as the vector. The genomic library (provided by J. Baum and R. Geever, University of Georgia) consisted of six independent pools, each derived from 10^4 phage plaques with λ-EMBL3 as vector. Hybridizations to the cDNA and genomic libraries were carried out at 42-55 °C when using the yeast *rp51* gene as a probe, and at 62 °C when using the *crp-3* PCR fragment as a probe. The hybridization buffer consisted of 2 x SSC (0.3 M NaCl, 0.03 M Na_3citrate), 0.5% SDS, 0.1% bovine serum albumin, 0.1% polyvinylpyrollidone, 0.1% ficoll, 100 μg/ml of heparin, and 1 mM EDTA. Following screening of the cDNA libraries, the positive plaque which carried the longest cDNA insert was identified by re-screening 10 strongly hybridizing plaques with PCR, using the *crp-3* 3' primer paired with either of the *universal* or *reverse* primers which flank the cDNA insertion sites of vector. This clone was sub-cloned by M13-assisted plasmid release (pop-out), avoiding plaque purification and phage propagation (Short *et al.*, 1988). Following screening of the genomic libraries, λ-phage DNA was prepared (Maniatis *et al.*, 1982) from a single positive clone from each of

four sub-libraries. Restriction mapping and further subcloning of cDNA and genomic clones were carried out essentially as described by Maniatis *et al.*, (1982). Exo-nuclease III was used to generate deletion clones for sequencing (Henikoff, 1984). Dideoxynucleotide sequencing with Sequenase (USB) was carried out using either alkali-denatured double stranded plasmid DNA or single stranded DNA. Single stranded plasmid DNA was prepared from *E. coli* cells containing Bluescript (Stratagene) or pTZ18R plasmids carrying the f1 ori site, by superinfection with M13K07 helper phages. Chemical sequencing was conducted according to Maxam and Gilbert (1980) with a modification (Shi and Tyler, 1989).

5.2.3. 5' End-mapping of *crp-3* mRNA. Transcription initiation sites were determined by S_1 nuclease digestion of DNA.mRNA hybrids, and by primer extension. The DNA probe used for S_1 mapping was a 175 bp *ApaLI-StyI* fragment spanning the 5' upstream and first exon regions. The primer was a 30 bp *BstUI-StyI* fragment within the first exon. S_1 mapping and primer extension assays were carried out essentially as described (Tyler and Harrison, 1990) except that hybridization was carried out at 50 °C instead of 56 °C for S_1 mapping.

5.2.4. Genetic mapping of *crp-3*. The genomic location of the *crp-3* gene was mapped using restriction fragment length polymorphisms

(RFLPs). The probe used for mapping was prepared from a 20 kb genomic clone by random primer labeling. One fragment was found to be polymorphic after *StyI* digestion of genomic DNAs of Oak Ridge (OR, 3.5 kb fragment) and Mauriceville (M, 3.8 kb fragment) strains of *N. crassa*. The location of *crp-3* was determined by comparing the segregation of M and OR alleles of *crp-3* among 38 progeny with the known segregation of 55 other RFLP loci (Metzenberg *et al.*, 1985).

5.2.5. Isolation and measurement of level of mRNA of *crp* genes. Conidia from each strain were shaken at 300 rev./min at 30 °C overnight without light in 800 ml of minimal Vogel's medium plus 1.5% sucrose to allow germination and outgrowth. After 13.5 hours, each culture was harvested by filtration onto Miracloth (Calbiochem), rinsed with ice-cold sterile distilled water, and then divided into three equal portions. Portion 1 (S3) was cultured in 1.5% sucrose medium for 3 hours. Portion 2 (Q3) and portion 3 (Q10) were cultured in minimal Vogel's medium plus 0.3% quinic acid (Giles *et al.*, 1985) for 3 and 10 hours respectively under the original conditions. Finally, the mycelia were harvested by filtration onto miracloth, rinsed with ice-cold sterile distilled water, frozen immediately in liquid N_2 and stored at -80 °C. mRNA was prepared from the frozen mycelia using the methods similar to those of Patel *et al.* (1981).

S1 nuclease mapping was used to measure the mRNA levels of four

different r-protein genes and the β-tubulin gene. The method used for S1 mapping was the same as that for mapping the 5' end of *crp-3* mRNA, except that the hybridizations were carried out at 58 °C. The fragments used for the S1 analysis were 580 bp *BstBI-EcoNI* (5' end labelled) for *crp-1* (Kreader and Heckman, 1987), 213 bp *EcoRI-BglII* (5' end labelled) for *crp-2* (Tyler and Harrison, 1990), 373 bp *BamHI-AvaI* (5' end labelled) for *crp-3*, 365 bp *EcoO109I* fragment (3' end labelled) for *crp-4* (a homolog of yeast *YL3*, de la Serna, Shi and Tyler, unpublished), and a 353 bp *PvuII-BamHI* (3' end labelled) for the β-tubulin gene (Orbach *et al.*, 1986). The mRNA level for each gene was measured by scanning the resulting radioactive band in the dried gels using an AMBIS radioanalytic imaging system (San Diego, CA).

5.3. RESULTS

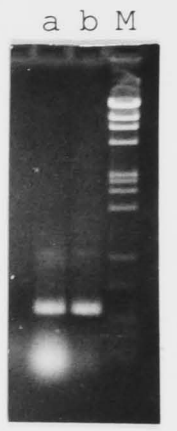
5.3.1. Cloning and sequencing of crp-3. The yeast *rp51* protein sequence is strongly homologous to the human *S17* protein, primarily at the N-terminus (Chen *et al.*, 1986). Initially, it was tried to clone the homologous gene in *N. crassa* by cross hybridization using the yeast gene as a probe, but were unsuccessful even using low stringency hybridization conditions. The failure to detect the gene could have been due to

FIG. 1. PCR amplification of *N. crassa crp-3* sequences. (*A*). Highly conserved regions of the yeast *rp51* and human *S17* proteins used to design PCR primers for cloning *crp-3*. The degenerate primer sequences were derived according to the biased codon usage of highly expressed *N. crassa* genes (Tyler and Harrison, 1990). (*B*) *Crp-3* DNA fragment (370 bp) amplified from *N. crassa* genomic DNA and resolved on a 1.5% agrose gel. Lane a: 10 μM of each primer; lane b: 1 μM of each primer; lane M: DNA size marker.

A

rp51/S17 N-terminus		M_1	G	R	V	R	T	K	T	V_9
crp-3 5' primer	5' ATT	GGT C	CGT C	GTT C	CGT C	ACT C	AAG	ACT C	GT	3'
rp51/S17 C-terminus		D_{92}	L	A	S	V	E	P	V	Y_{84}
crp-3 3' primer	5' GTC	AAG G	AGC G	AGA G	AAC G	CTC	GGG	AAC G	ATA G	3'

B



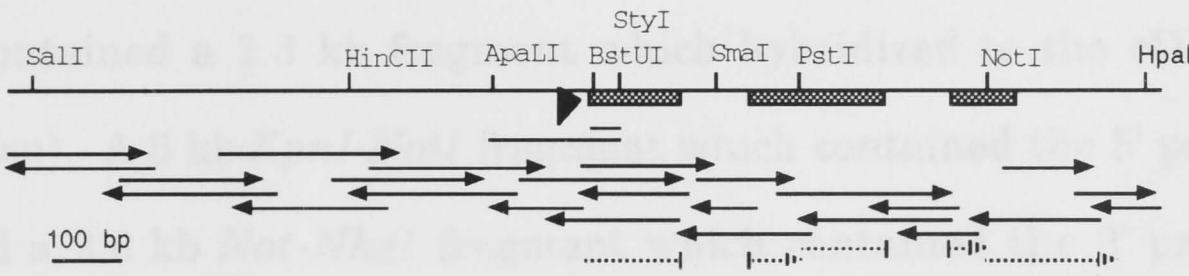
differences in the codon usage between yeast and *N. crassa*. Instead, a PCR strategy was used to clone the gene. The primers for PCR were synthesized based on two highly conserved regions of the yeast *rp51* and human *S17* r-protein sequences (Fig 1A). The degenerate primers were based on the most common codon usage in several *N. crassa* genes (Fig 1A). PCR amplification with these primers resulted in a specific DNA fragment of about 370 base pairs (Fig 1B).

The amplified genomic fragment was used as a probe for screening the cDNA libraries. Many strongly hybridizing plaques were identified from screening each cDNA library. A single clone with the longest insert (750 bp) was identified by PCR screening of 10 strongly hybridizing plaques. The insert was subcloned by M13-assisted plasmid release, then sequenced. The 5' end of the cDNA sequence (the first 276 bp of the coding region) showed 74% homology with yeast *rp51* and 75% with human *S17*. A part of the cDNA 5' sequence (103bp) which had no homology with the yeast *rp51* gene later proved to be a cloning artifact by comparison with the sequences of genomic clones (not shown) and by 5' end mapping of the mRNA.

To clone the *crp-3* gene, each pool from the genomic library was screened with the PCR amplified genomic fragment. More than 20

FIG 2. Sequencing strategy and nucleotide sequence of the *crp-3* gene. (*A*) Sequencing strategy. The black arrow head indicates the transcription initiation site. Exons are shown by the shaded boxes. Solid lines indicate the hybridization probe and primer used to map the 5' end of the *crp-3* mRNA. The solid arrows indicate sequencing of the gene using Exo-III deletions. The dashed arrows indicate sequencing of the cDNA region using restriction site deletions. (*B*) Nucleotide sequence of the *crp-3* gene. The triangle indicates the major transcription start point and the diamonds indicate the minor starts (see Fig 3). Slashes show the intron boundaries. Underlined sequences in the introns are conserved heptamers found in *N. crassa* introns (Rambosek and Leach, 1987). Asterisks indicate identities between the predicted *crp-3* protein sequence and the yeast *rp51* and human *S17* protein sequences. The reversed double arrow at the 3' end marks the polyadenylation site in the cDNA clone. The full *crp-3* 5' flanking sequence is shown in Fig 6. The Genbank accession number for this sequence is M63879.

A



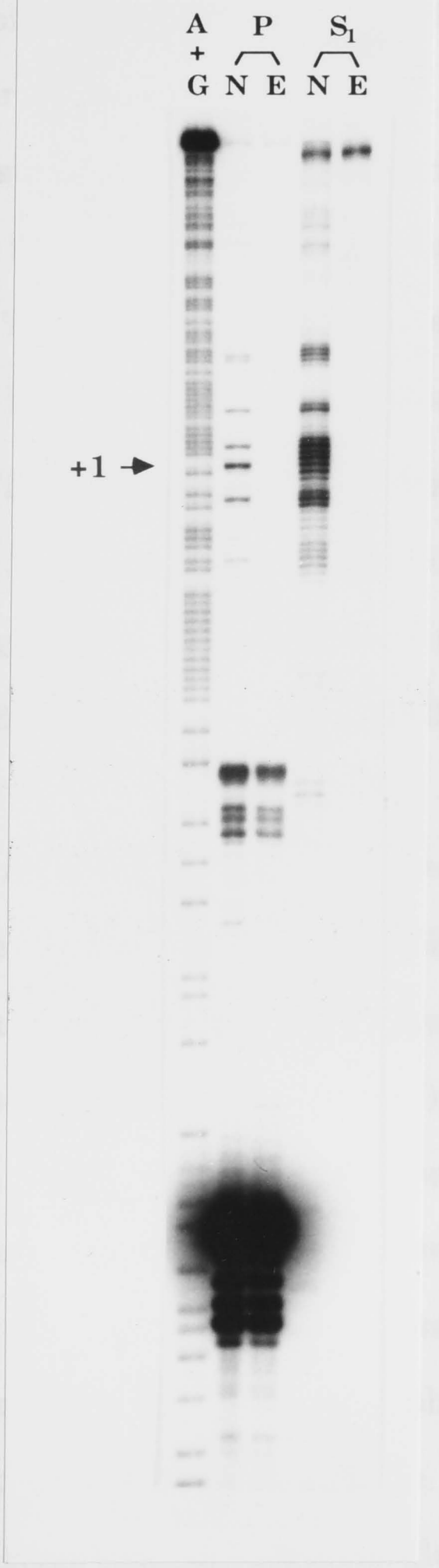
B

```
-23                          +1                                                                                    75
TGTTCACGTC TTCTTGTCCT TGACAACGTA ACCCGTCAAC TCCCCTCCTT CAGCAGCAAA GCAGCAGCAA AGCCGGCAAA ATG GGT CGC GTT CGT ACC
   ◊◊          ◊      ◊ Δ   ◊        ◊                                                    M   G   R   V   R   T
                                                                                HOMOLOGY TO RP51:   *   *   *   *   *   *
                                                                                HOMOLOGY TO S17:    *   *   *   *   *   *

                                                                                                                   159
AAG ACT GTC AAG AAG TCC GCC AAG GTC ATC ATT GAG CGG TAC TAC CCC AAG TTG ACC CTC GAC TTC GAG ACC AAC AAG CGC ATC
 K   T   V   K   K   S   A   K   V   I   I   E   R   Y   Y   P   K   L   T   L   D   F   E   T   N   K   R   I
 *   *   *   *           *       *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
 *   *   *   *   *       *       *   *   *   *   *       *   *       *       *   *   *   *   *   *   *

                                                                                                                   250
TGC GAT GAG ATC GCC ATC ATT GCC TCC AAG CGC CTC CGC AAC AAG /GTGCGTTTTT GTCACGAACC GGGAGAGGGT TTATATATGG ACCCGG
 C   D   E   I   A   I   I   A   S   K   R   L   R   N   K
 *   *   *   *   *       *       *   *   *   *   *   *   *
 *       *   *   *   *   *       *   *       *   *   *   *

                                                                                                                   342
GCGGAAGGAC AGACAGAAGG GGGATCTAAC ATGTTTGACA CCAATAG/ ATT GCC GGC TAC ACC ACC CAC TTG ATG AAG CGT ATC CAG CGT GGC
                                                     I   A   G   Y   T   T   H   L   M   K   R   I   Q   R   G
                                                     *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
                                                     *   *   *   *       *   *   *   *   *   *   *   *   *   *

                                                                                                                   426
CCC GTC CGC GGT ATC TCC TTC AAG CTG CAG GAG GAG GAG CGT GAG CGC AAG GAC CAG TAC GTT CCC GAG GTC TCT GCT CTC GAC
 P   V   R   G   I   S   F   K   L   Q   E   E   E   R   E   R   K   D   Q   Y   V   P   E   V   S   A   L   D
 *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
 *   *   *   *   *   *       *   *   *   *   *   *   *   *   *       *       *   *   *   *   *   *   *   *

                                                                                                                   511
TTC ACC CAG AAC TCC GAG AGC GGT CAG CTC GAC GTC GAC ACC GAG ACC AAG GAC CTC CTC AAG CAC CTC GGC /GTAAGTTTCC TTC
 F   T   Q   N   S   E   S   G   Q   L   D   V   D   T   E   T   K   D   L   L   K   H   L   G
             *       *       *       *   *               *       *   *       *       *   *
                                     *   *               *   *           *   *       *

                                                                                                                   608
ACTATCACAC CCAACCATCA CTGGTTTTCT GGCGCGCATG AAGCCATGGT AAGGATTTCA TTTTGTCTAA CTTACCTTCG AACAG/ TTC GAC TCC ATC
                                                                                                F   D   S   I
                                                                                                *       *

                                                                                                                   691
CCC GTC AAC GTC ATC CCC GTC ACC CAG GCC CAG CCT GTC GAG CGC GGC CGC CGC TTC GGC GAC CGT CCC CGC CGC GAC /TAAAT
 P   V   N   V   I   P   V   T   Q   A   Q   P   V   E   R   G   R   R   F   G   D   R   P   R   R   D
 *           *   *       *           *   *               *       *   *               *
                         *   *   *

                                                                                                                   791
GTCGCCCGGT CGACGATAAC CAAAACAATA ATGGGAAATC GGGCGTTGGA TCATGCACTA CGGTGGTGTC GGTCCTACCT GGGTTGGTGT TCGGTGTCTA

                                                                                                                   891
TCTGGAACAG GGTAGCATGA GAGCATCATT AGGTCACATC TGCGAAACCA AATGCGATCT GTTTGCCCAC AAAAGGTGTC GCTGACTCTC TTGGTGTAAT
                                                                                                »

GCCGGAGACG AT
```

strongly hybridizing plaques were identified. Four positive plaques, each from an independent pool, were selected and purified for further analysis. Restriction digestion and Southern analysis indicated that all of the four clones contained a 1.3 kb fragment which hybridized to the cDNA clone (not shown). A 5 kb *KpnI-NotI* fragment which contained the 5' part of the gene and a 2.6 kb *Not-NheI* fragment which contained the 3' part of the gene were subcloned into the Bluescript plasmid for sequencing.

The sequencing strategies and the sequences of the cDNA and genomic clones are shown in Fig 2A and 2B. The introns were identified by comparing the cDNA sequence with the genomic sequence. Unlike the yeast *rp51* gene, which contains only one intron, the *N. crassa* gene possesses two introns and three exons. Both intron sequences start with GT and end with AG. An 8 bp consensus sequence common to *N. crassa* introns (Rambosek and Leach, 1987) was also found in each intron (underlined in Fig 2B). The inferred protein sequence of the *crp-3* gene shares 89% homology with the yeast *rp51* protein and 83% homology with the human *S17* protein at the N–terminus, as is the case with yeast *rp51* and human *S17* proteins (Chen *et al.*, 1986). This suggests that the *N. crassa* gene is a homolog of both genes. Since this is the third *N. crassa* r-protein gene characterized, it was named *crp-3* (*c*ytoplasmic *r*ibosomal *p*rotein). The polyadenylation site of the *crp-3* cDNA clone is located in a purine rich region (marked by the reverse arrow head in Fig 2B).

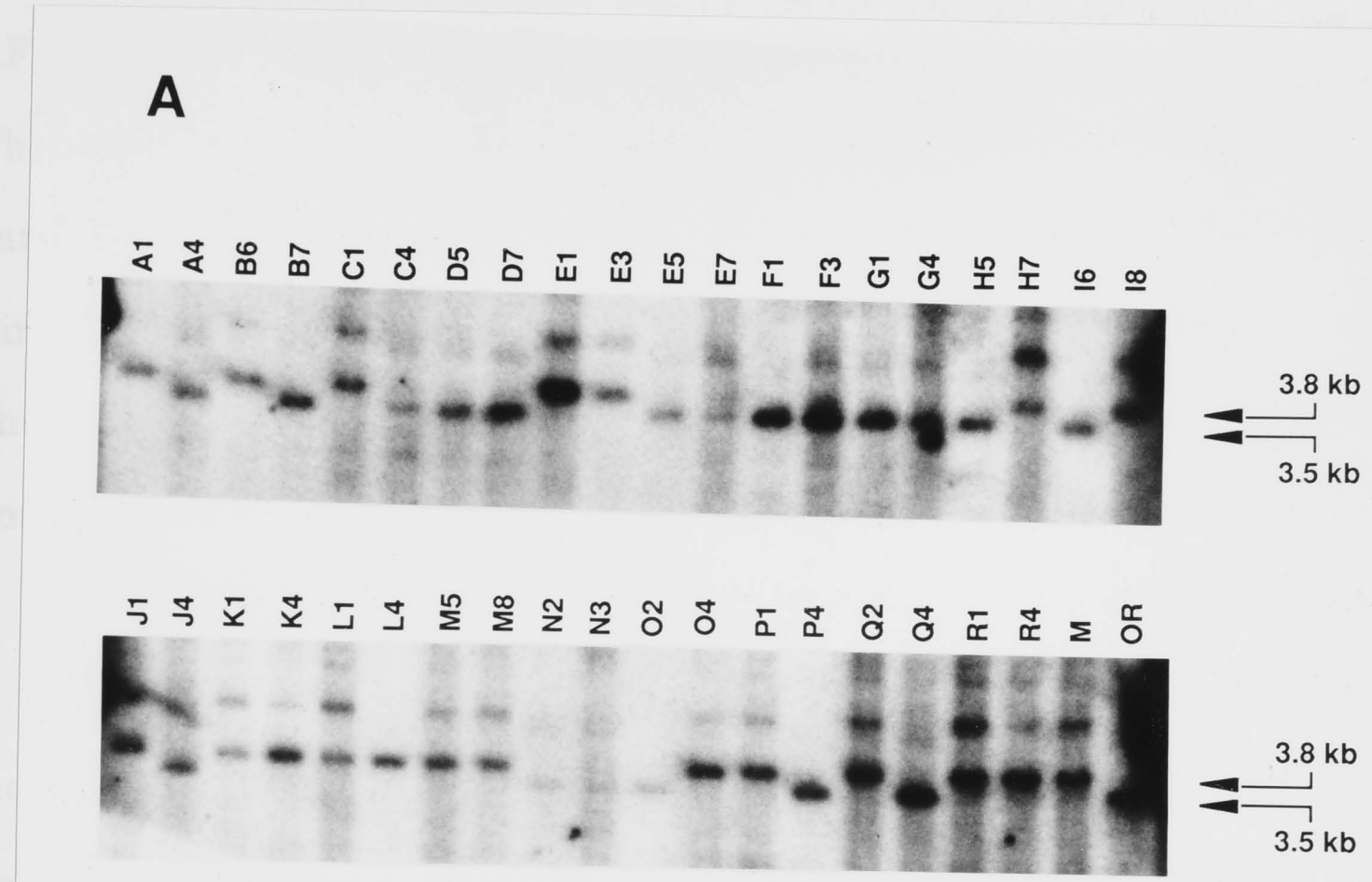
FIG. 3. Mapping of the transcription initiation site of *crp-3* by primer extension and S1 nuclease mapping. The major transcription site is indicated by the arrow as the +1 site. The lanes are A+G: A+G chemical sequencing of S_1 probe as marker; P: primer extension; S1: S1 nuclease mapping; N: *N. crassa* mRNA, and E: *E. coli* control RNAs.


A
+
G
P
N
E
S1
N
E
+1

The transcription initiation sites of the *crp-3* gene were mapped by S_1 nuclease digestion of RNA.DNA hybrids and by primer extension. The DNA probe used for S1 mapping was a 175 bp *ApaLI-StyI* fragment spanning the 5' upstream and first exon regions. A 30 bp *BstUI-StyI* fragment within the first exon was used for primer extension. The results are shown in Fig 3. The major initiation site was mapped at -57 relative to the first AUG codon by both S_1 and primer extension assays (indicated by the arrow head in Fig 3). A total of 10 initiation sites were revealed in a region between 107 to 43 bp upstream from the first AUG codon. It is common in fungi that the 5' ends of mRNAs are heterogeneous (Struhl, 1989; Tyler *et al.*, 1984; Tyler and Harrison, 1990).

5.3.2. Genetic mapping of the crp-3 gene. Gel blot hybridization analysis using the *crp-3* cDNA as a probe showed a single band hybridizing to *N. crassa* genomic DNA digested with three different restriction enzymes (*BamHI*, *SalI*, and *PstI*), indicating that the *crp-3* gene is present in a single copy (data not shown). This differs from yeast where the *rp51* gene is present in two copies (Teem *et al.*, 1983). To genetically map the *crp-3* gene, a 20 kb genomic fragment containing the *crp-3* gene was used as a hybridization probe to screen for restriction fragment length polymorphisms (RFLPs) in genomic DNAs of two *N. crassa* strains, Oak ridge (OR) and Mauriceville (M). A *StyI* fragment (3.8 kb in *M* and 3.5 kb in *OR*) was found to be polymorphic (lane *OR* and *M*, Fig

FIG. 4. Genetic mapping of *crp-3* using RFLPs. (*A*) Segregation of an RFLP adjacent to the *crp-3* gene. DNAs from 38 progeny of an Oak Ridge (OR) x Mauriceville (M) cross (Metzenberg and Groteluschen, 1987) were digested with *StyI* and hybridized with a 20 kb genomic fragment carrying the *crp-3* gene. The polymorphic fragments from the digestion are indicated by the arrows. OR and M indicate DNA from the Oak Ridge and Mauriceville parents respectively. A1, A4, B6 etc indicate progeny DNAs. Progeny with same letter code (e. g. A1 and A4) originate from the same ordered tetrad (Metzenberg and Groteluschen, 1987). (*B*) The RFLP map containing *crp-3*. The circle indicates the centromere. The Greek letters represent 5S rRNA genes. *Crp-3* was aligned at the same location as the *cot-1* locus, but due to uncertainties in the mapping, they may be several cM apart.


A
A1
A4
B6
B7
C1
C4
D5
D7
E1
E3
E5
E7
F1
F3
G1
G4
H5
H7
I6
I8
3.8 kb
3.5 kb
J1
J4
K1
K4
L1
L4
M5
M8
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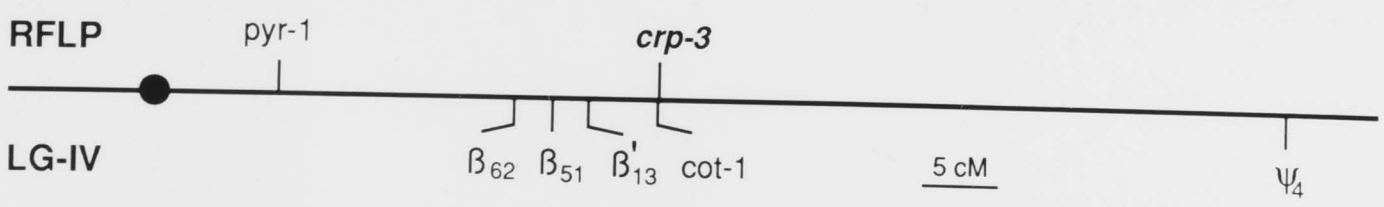
Q4
R1
R4
M
OR
3.8 kb
3.5 kb

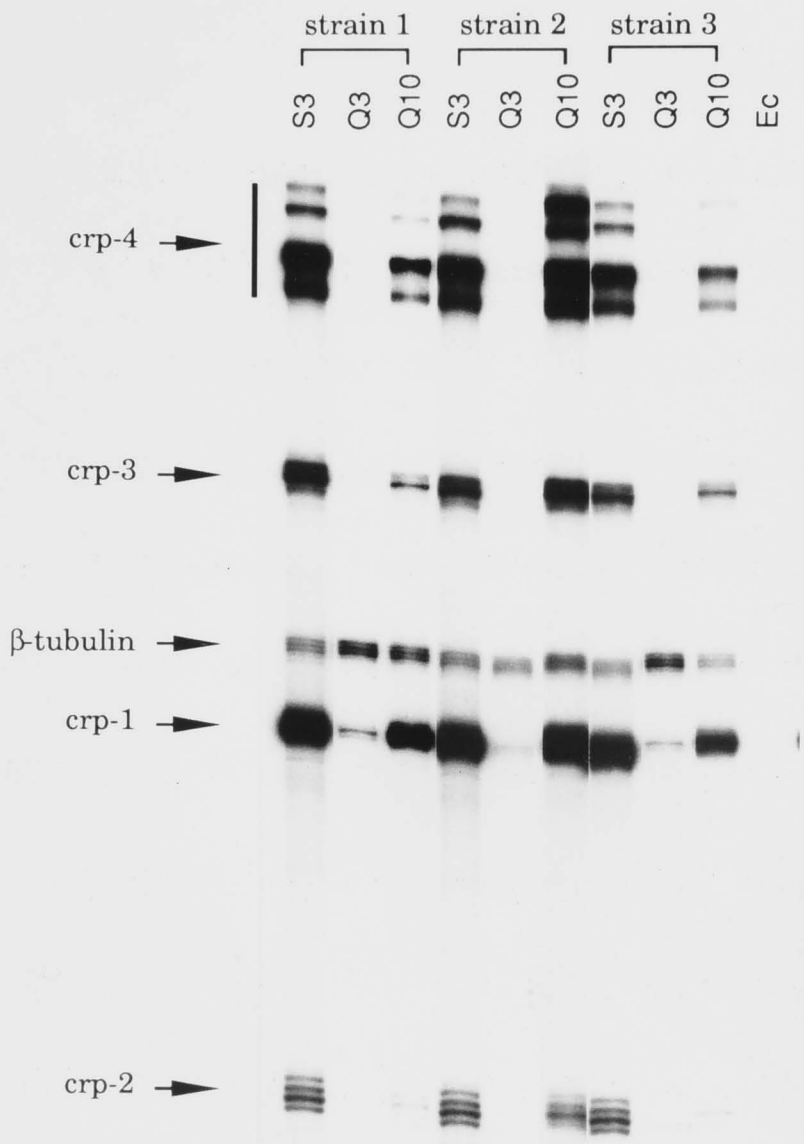

B
RFLP
pyr-1
crp-3
LG-IV
ß62
ß51
ß'13
cot-1
5 cM
ψ4

4A). The 20 kb probe was then hybridized to *StyI* digested DNAs from 38 progeny of a cross between the Oak Ridge and Mauriceville strains (*lane A1* to *R4*, Fig 4A). This progeny set has previously been used to construct RFLP genetic maps of *N. crassa* (Metzenberg and Groteluschen, 1987). The *crp-3* gene was mapped to the right arm of linkage group *IV* at the same location as the *cot-1* locus (Fig 4B) but *crp-3* and *cot-1* have not been directly mapped with respect to each other. Sequence comparison showed that *crp-3* and *cot-1* are distinct genes (C. Yanofsky, personal communication).

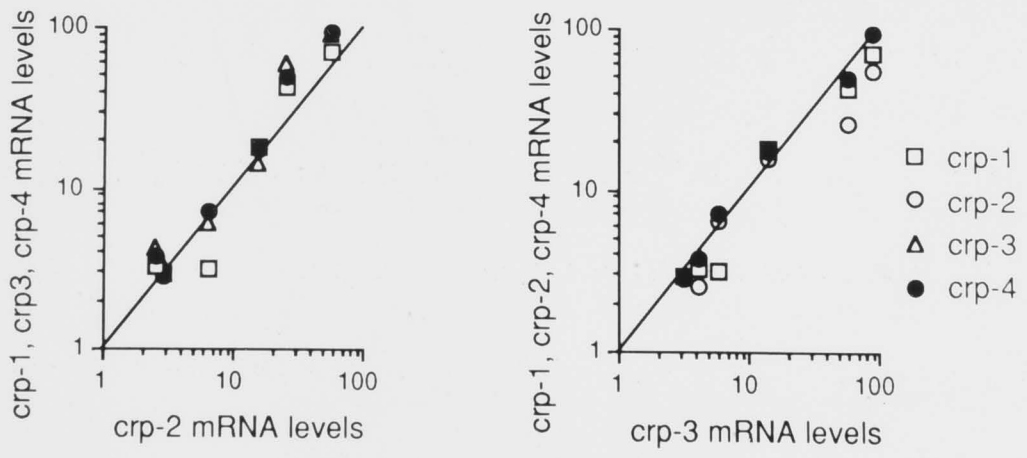
5.3.3. Coordinate changes in r-protein mRNA levels during a nutritional downshift. To begin to examine the regulation of *N. crassa* r-protein gene expression, and in particular to determine the level of coordinate control, the mRNA levels of four *N. crassa* r-protein genes were measured at several points during a nutritional downshift. Three strains derived from 246-89601-2A by transformation (Chapter 4) were grown to mid log phase in sucrose medium, then the mycelia were harvested and divided into three portions. The portions were reinoculated into 1.5% sucrose for 3 hr (S3), into 0.3% quinic acid for 3 hr (Q3) or into 0.3% quinic acid for 10 hr (Q10). Quinic acid is a poor carbon source for *N. crassa* and adaptation to stable growth on quinic acid takes 4-6 hr (Giles *et al.*, 1985). In each culture, the mRNA levels were measured for r-protein genes *crp-1* (Kreader and Heckman, 1987), *crp-2* (Tyler and Harrison, 1990), *crp-3* (this

FIG. 5. Coordinate regulation of r-protein mRNAs during a carbon nutritional downshift (*A*) Quantitation of mRNAs by S1 nuclease digestion of RNA.DNA hybrids. The mRNA was isolated from three *N. crassa* strains grown in sucrose for three hours (S3), or in quinic acid for 3 hours (Q3) and 10 hours (Q10) respectively. Ec indicates control RNA from *E. coli*. The DNA probes used were fragments from the *crp-1*, *crp-2*, *crp-3*, *crp-4*, and β-tubulin genes (see Material and Methods). The respective fragments protected from S1 nuclease are marked by the arrows. The multiple bands (marked by a vertical line) from *crp-4* reflect multiple termination sites of transcription. (*B*) Correlation of relative mRNA levels among the *crp* genes during the downshift. All data were derived from quantitation of the radioactive bands shown in the autoradiograph and were standardized with the mRNA level of the β-tubulin gene. The relative mRNA levels were expressed as a percentage of the level in sucrose cultures. The levels of the three other *crp* genes were plotted against *crp-2* (on the left) or *crp-3* (on the right). The diagonal lines on each graph indicate the points at which the three mRNA levels (y-axis) are identical to the fourth (x-axis).

A


strain 1
strain 2
strain 3
S3
Q3
Q10
S3
Q3
Q10
S3
Q3
Q10
Ec
crp-4
crp-3
β-tubulin
crp-1
crp-2

B


crp-1, crp3, crp-4 mRNA levels
100
10
1
1
10
100
crp-2 mRNA levels
crp-1, crp-2, crp-4 mRNA levels
100
10
1
1
10
100
crp-3 mRNA levels
crp-1
crp-2
crp-3
crp-4

paper) and *crp-4* (de la Serna, Shi and Tyler, unpublished). *Crp-4* was cloned by homology to the yeast 5S rRNA-binding r-protein *YL3* using PCR (Shi and Tyler, unpublished). The preliminary sequence of a *crp-4* cDNA clone encodes an amino acid sequence with 68% identity to that of yeast *YL3* (de la Serna, Shi and Tyler, unpublished; J. Woolford, personal communication). ß-tubulin mRNA levels were measured as a standard. S1 nuclease mapping was used to quantitate simultaneously all five mRNAs in the same hybridization reaction. The results are shown in Fig 5A. In each strain, after 3 hours growth in quinic acid all four r-protein mRNAs had dropped sharply to 3-5% of the sucrose grown levels (compare lane S3 with Q3 in Fig 5A), while the ß-tubulin mRNA levels remained about the same. After 10 hrs in quinic acid (Q10 in Fig 5A), each of the r-protein mRNA levels had partially recovered. The extent of recovery varied among the three strains (20% to 80% of the S3 levels). However, the relative levels of the four r-protein mRNAs remained closely coordinated. Fig 5B shows the relative levels of the *crp-1*, *crp-3* and *crp-4* mRNAs plotted against *crp-2* for all three strains after 3 and 10 hr in quinic acid (on the left). The levels of *crp-1*, *crp-2* and *crp-4* mRNAs are also plotted against *crp-3* in a similar manner (on the right). In each case the mRNA levels are expressed as a percentage of the level in the sucrose cultures, after normalization to the ß-tubulin mRNA level. In all cases there is a close coordination of the four r-protein mRNA levels irrespective of the absolute levels.

FIG. 6. Conserved and repeated sequences in the *crp-3* promoter region. Closed rectangles indicate sequences shared among all three *crp* genes in *N. crassa*. The stippled thick arrow shows sequences conserved between *crp-3* and *crp-1*. The two striped thick reversed arrows demonstrate sequences shared between *crp-3* and *crp-2*. The dashed rectangles point out two pyrimidine rich regions around the transcription initiation site (indicated by triangles as +1). Two direct repeat sequences are indicated by two different thin arrows.

-791 -741

CGGTGCC AAGCTCTGGG TCAACGTTGA CAAGGCGACG GTCGACTCGA CTGGCCGCGT ACAGCTGACT GTTAGTGATG

-691 -641

CCAAGGCGAC GGATGTTCTC ATCGCCAAAG AATTGATTGG CGACGCCCGT GAGCCCATCT TCGGGTCTGC CCCCAAGCGC CAGGAAAAGA CCTTCAAGAA

-591 -541

GATCCCTATG ACTGCTGCAA GGCTGAGGGG CAGGTACGAG AACTCTGACT CGCCCTCATC ACCTACCATG TCGGGCTCGT CTGGGTATGG TTTACGGTAA

-491 -441

ACCGGCCGAT AGGCAGAAGT GGGAACACGA ATGCTTCGCG GCGAGCAGTC AATGAGATAC CCAAAATCTG GCAAGAAGAC TGGAGGGACG CGATTGGGAT

-391 -341

CTTTGCAGGC CGTGTAGGTG TGGATAGCTG ATGGTCATAC TTATCACCGC TCGCAAATCA GTCATAAAAC AGACAGTCCA TTGTAACAAT CTAAGCTGTA

crp-2(-361)

-291 -241

AATATAGACC GGTTCATAAA GACCTCCTGC GATGAGATGC GTTGACATTC TTGAAAtCTt GgcGATCGAG AGCTGAGTAG TTGTTTAAGG GTAAGTGAGT

RIBO BOX *crp-1(-237)*

-191 -141

GTCACTGCCT TTGTACTGTA AGCGTTCGAT CGGTTCAGGG GCAACACTGA GTGTAAACAA TGGGGGTCAC GTGCCGAGCG GACCATGACC ACTCAGCCAC

crp-2(-150)

-91

GACACTCCTC AGCCACATGA GGCTCAGTAC GTGCCCTAAC GCCAAACCTC AGCTGGGCGT GCACTGACCC CACAACCCGC CGGATATTGC CAGACCAAAA

crp-2(-150) *crp-2(-116)* **CG-REPEAT**

+1 +50

AAGTTCGACT TGGTGTTTGT TCACGTCTTC TTGTCCTTGA CAACGTAACC CGTCAACTCC CCTCCTTCAG CAGCAAAGCA GCAGCAAAGC CGGCAAAATG

TAQ BOX Met

5.3.4. Promoter elements common to r-protein genes in N. crassa. The *crp-3* promoter sequence was compared to the *crp-1* and *crp-2* promoter sequences to reveal any common elements, like UAS_{rpg} in yeast, which may potentially coordinate transcription of the r-protein genes. In particular, the *crp-3* promoter was searched for the presence of the Ribo box which is found three times in the *crp-2* promoter (Tyler and Harrison, 1990) as well as in the 5S and the 40S rRNA genes. Fig 6 summarizes the comparisons. No sequences with better than 4 mismatches to the Ribo box were found in the *crp-3* promoter. One sequence with 4 mismatches was found at -291 (boxed at -291 in Fig 6), in comparison to the *crp-1* promoter region which carries 3 sequences with 4 mismatches to the Ribo box (Tyler and Harrison, 1990). Two sequence elements were found to be common, in sequence and position, among the three r-protein genes (sequences in solid box, Fig 6). One is an element named the *Taq* box (it contains a *TaqI* restriction site in *crp-2*) which is a 10 bp partially palindromic sequence with a consensus of ARTTYGACTT (R: purine, Y: pyrimidine). This element is located at -39 in the *crp-3* promoter, at -39 in *crp-2* in the opposite orientation, and at -40 in *crp-1* (Fig 7*A*). The other element was previously identified in *crp-1* and *crp-2* and named the CG repeat, with the consensus CCCRCCRRRT (Tyler and Harrison, 1990). It is located at -65 in *crp-3*, at -192, -74 and -26 in *crp-2*, and at -66 in *crp-1* (Fig 7B). In addition, pair wise comparisons identified one 8 bp sequence perfectly conserved between *crp-3* and *crp-1* (thick stippled arrow in Fig 6), and two different 8 bp elements

perfectly conserved between *crp-3* and *crp-2* at similar locations but in opposite orientations (the thick reverse arrows in Fig 6). The *crp-3* promoter contains a 9 bp perfect direct repeat in the 5' flanking region at -149 and -133 (the broken thin arrows in Fig 6). This sequence also occurs at -150 in *crp-2* (with one mismatch) and possibly in *crp-1* (at -231 with two mismatches) giving a consensus of CTYAGCCRY. There is a second perfect direct repeat of 12 bp at +28 and +39 in tandem, 7 bp upstream from the first AUG codon (the solid thin arrows in Fig 6). The *crp-3* promoter does not possess a TATA-like sequence, nor does it have any matches to the yeast UAS_{rpg} sequence.

5.4. DISCUSSION

In *Neurospora crassa*, the syntheses of rRNAs and r-proteins are coordinately regulated during nutritional shifts (Alberghina and Sturani, 1981). However, little is known about the mechanism involved in coordination, either at the transcriptional or translational level. To facilitate the studies on mechanisms involved in co-regulation of ribosomal genes in *N. crassa*, the *crp-3* gene of *N. crassa* was cloned and sequenced. This is the third r-protein gene identified in this fungus. The N-terminus of the inferred *crp-3* protein sequence shows 89% homology with yeast *rp51* and 83% homology with human *S17*, as is the case between the *rp51* and the *S17* proteins (Chen *et al.*, 1986). *Crp-3* is a single copy gene, like *crp-1*

and *crp-2*, in contrast to the *rp51* gene and many other yeast r-protein genes which are duplicated in the genome (Warner, 1989). *Crp-3* contains two introns, none of which are conserved in position with the yeast *rp51* gene which carries only one intron (Teem and Rosbash, 1983), nor with the human *S17* gene which possesses four introns (Chen and Roufa, 1988). The first intron of human *S17* is conserved in position with that of yeast *rp51*. In the *N. crassa* *crp-2* gene, two intron positions are conserved in the human *S14* gene but no intron positions are conserved between the yeast *rp59* and human *S14* genes (Tyler and Harrison, 1990). In *crp-2* and *crp-3*, no intron positions are conserved in the *N. crassa* and yeast genes, though *N. crassa* and yeast are evolutionarily closer to each other than to humans.

The mRNA levels of four r-protein genes (*crp-1*, *crp-2*, *crp-3* and *crp-4*) were measured during a nutritional downshift from growth on sucrose to growth on quinic acid. Quinic acid is a poor carbon source for *N. crassa* and adaptation to stable growth on quinic acid takes 4-6 hr (Giles *et al.*, 1985). The levels of all four mRNAs dropped sharply to about 3-5% of the sucrose levels after 3 hrs culture in quinic acid. After a total of 10 hr in quinic acid, the levels of the four r-protein mRNAs had recovered to about 20%, 80%, or 50% of sucrose levels in strain 1, 2, and 3 respectively. These results show that r-protein mRNA levels in *N. crassa* are regulated during a carbon downshift. Presumably the mRNA levels are responding

to a change in the growth rate (Alberghina and Sturani, 1981), or to signals from the carbon catabolite regulatory system (Geever *et al.*, 1989). An *N. crassa* r-protein mRNA homologous to rat *S26* also was reported recently to be regulated by carbon nutrition (Tarawneh *et al.*, 1990). The results also showed that there was close coordination of the relative levels of the four r-protein mRNAs during the downshift. This was particularly apparent when the r-protein mRNA levels in the three strains were compared after 10 hr in quinic acid; although the absolute mRNA levels in the three strains had recovered to different levels, the relative levels of the four r-protein mRNAs were very similar within each strain.

The rates of rRNA synthesis in *N. crassa* have been measured during a carbon downshift from glucose to glycerol (Sturani *et al.*, 1973). The rate of rRNA synthesis dropped to 10–15% of glucose grown levels during the downshift, and remained at that level for 2 hr (Sturani *et al.*, 1973). After 3 hr, the rate of rRNA synthesis recovered to about 50% of the glucose grown level and remained at that level until the end of the experiment at 6 hr (Sturani *et al.*, 1973). These changes in the rate of rRNA synthesis closely parallel the changes in r-protein mRNA levels observed. Therefore, in *N. crassa*, the levels of r-protein mRNAs appear to be down-regulated in concert with the rate of rRNA synthesis during a carbon downshift. However, it is not clear presently whether the r-protein mRNA levels are regulated by changes in the stability of the mRNAs or by changes in the

transcription rate. The rate of *N. crassa* rRNA synthesis also drops to 3-4% of the control during phosphorus starvation (Stellwag and Metzenberg, 1984). It will be of interest to measure r-protein mRNA levels during phosphorus starvation, as well as other types of nutritional shifts.

In *Saccharomyces cerevisiae*, the transcription rates of most r-protein genes are coordinately regulated by growth rate and carbon nutrition by the action of a common upstream transcriptional element, the UAS_{rpg}. The UAS_{rpg} is a 15 bp conserved sequence often found in duplicated copies or in tandem repeat 250-450 upstream of the first AUG codon of r-protein genes (Warner, 1989). Deletion of the UAS_{rpg} usually results in a reduction of transcription of a gene carrying the sequence (Larkin *et al.*, 1987; Rotenberg and Woolford, 1986; Schwindinger and Warner, 1987; Woudt *et al.*, 1986). The UAS_{rpg} is also found in the 5' flanking region of non-ribosomal protein genes (Buchman *et al.*, 1988a; Buchman *et al.*, 1988b), while some r-protein genes, like *L3* and *S33,* are not co-regulated via the UAS_{rpg} (Mager, 1988; Warner, 1989). Therefore, the UAS_{rpg} is not confined to coordinating r-protein genes. In addition, little is known about how transcription of yeast r-protein genes is coordinated with rRNA synthesis.

FIG. 7. Consensus sequence for the Taq box (*A*) and the CG repeat (*B*). R: purine; Y: pyrimidine. > and < indicate relative orientations. Lower case letters represent mismatches with the consensus.

A

TAQ BOX

crp-1	-40	AATTTGACTT	-31	>
crp-2	-30	AGTTCGACTT	-39	<
crp-3	-40	AGTTCGACTT	-31	>
consensus		ARTTYGACTT		

B

CG REPEAT

crp-1	-66	CCCACCAAAa	-57	>
crp-2	-74	CCCGCCAAGT	-65	>
crp-3	-65	CCCGCCGGAT	-56	>
crp-2	-192	CCCGCgAAAT	-183	>
crp-2	-26	CCCGCCGAAT	-17	>
consensus		CCCRCCRRRT		

To search for a conserved sequence element which might be responsible for the coordinated transcription of r-protein genes in *N. crassa*, computer comparisons were carried out among the 5' flanking sequences of the *crp-1*, *crp-2*, and *crp-3* genes. The analysis revealed two highly conserved sequences located at about -39 and -65 in each of the three r-protein genes. The sequence at around -65 was previously described as common to *crp-1* and *crp-2* and was named the CG repeat (Tyler and Harrison, 1990). It is present at -65 in *crp-3*, at -66 in *crp-1*, and three times in *crp-2*, at -192, -74 and -26 (Fig 7B). The CG repeat resembles the human GC box (consensus: RYYCCGCCC$^{G}/_{T}$) which is known to be binding site for the transcription activator *SP1* (Courey *et al.*, 1989; Dynan and Tjian, 1985). The CG repeat in *crp-2* binds human *SP1* factor *in vitro* (Y. Shi and B. M. Tyler, unpublished), but it is unknown whether there is an *SP1* like factor in *N. crassa*. The conserved sequence at -39 is a partially palindromic sequence, designated the Taq box, which is present at -40 in *crp-1* and *crp-3*, and at -39 in *crp-2* but in opposite orientation (Fig 7A). The Taq box is relatively A-T rich and resembles in location the A-T rich sequence in mammalian r-protein genes, which is important for transcriptional efficiency *in vivo* (Hariharan and Perry, 1990).

Crp-3 lacks an obvious TATA box, like *crp-2* and many other genes in *N. crassa* (*crp-1* has a possible TATA box at -42). *Crp-3* also shares some

common features with *crp-1* and *crp-2* around the major transcription initiation site. In each case, the major initiation site is embedded in a relatively purine rich region and is flanked on the 5' side by a pyrimidine rich sequence, partially resembling the features of r-protein genes in mammalian systems. The transcription start site of the human *S16* r-protein gene is determined *in vivo* by a pyrimidine rich tract (Hariharan and Perry, 1990). In filamentous fungi such as *Aspergillus nidulans* and *N. crassa*, many genes contain a pyrimidine rich region upstream of their transcription initiation sites (Ballance, 1986; Gurr *et al.*, 1988; Hamer and Timberlake, 1987; Punt *et al.*, 1990). In the case of the *trpC* and *gpdA* genes of *A. nidulans*, the pyrimidine rich sequence is important for efficient transcription (Hamer and Timberlake, 1987; Punt *et al.*, 1990). It is possible that the conserved elements, including the CG repeat and the Taq box, serve as a common core promoter of r-protein genes in *N. crassa*, similar to mammalian r-protein genes (Dudov and Perry, 1986; Hariharan *et al.*, 1989; Hariharan and Perry, 1989). Confirming the role of the conserved sequences will require detailed mutational analyses of the promoters.

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